Cysteine Scanning Mutagenesis of the Segment between Putative Transmembrane Helices IV and V of the High Affinity Na+/Glucose Cotransporter SGLT1

EVIDENCE THAT THIS REGION PARTICIPATES IN THE Na+/AND VOLTAGE DEPENDENCE OF THE TRANSPORTER

Bryan Lo‡ and Mel Silverman§

From the Department of Medicine, University of Toronto, Toronto, Ontario M5S 1A8, Canada

Site-directed mutagenesis and chemical modification of specific cysteine amino acid side chains by methanethiosulfonate (MTS) derivatives were combined to elucidate structure/function relationships of the cloned rabbit Na+/glucose cotransporter, SGLT1. Each amino acid in the region (residues 162–173) between putative transmembrane helices IV and V of SGLT1 was replaced individually with Cys. Mutant proteins were expressed in Xenopus laevis oocytes and studied using the two-electrode voltage clamp method. At certain key positions, Cys substitution resulted in 1) a change in the apparent affinity for sugar, 2) an alteration in the voltage dependence of the transient currents, and 3) a sensitivity to inhibition by either the ethylamine (MTSEA) or the ethylsulfonate MTS derivatives. For the three Cys sensitivity to inhibition by either the ethylamine (MTSEA) and H+/oligopeptide (18, 19), the Na+/K+ ATPase (15), the Na+/Ca2+ exchanger (16), the H'/myo-inositol (17), the H'/oligopeptide (18, 19), the Na+/GABA (20, 21), the Na+/P(22), and the Na+/glucose cotransporters (2–4, 7, 23, 24). Although the kinetics and ion dependences of the transient currents differ from transporter to transporter, the unifying concept is that the transient currents reflect an interaction between the cation and the transporter, and/or a conformational change that moves protein charges or dipoles across the membrane electric field.

The sodium dependence, the effect of saturating concentrations of sugar and phlorizin, and the effect of temperature on the SGLT1 transient currents have all been studied (2–4, 24). With simulations of steady-state kinetic data using a six-state model (24) providing some of the kinetic parameters, the transient currents have been modeled by Loo et al. (4) as arising from two transitions, namely the reorientation of the empty transporter within the membrane and a subsequent external Na+ binding event. The transient currents exhibited by SGLT1 have also been studied using the cut open oocyte method (25), which allows for an ultrafast voltage clamp and access to the intracellular ion concentrations. Using this technique, the transient currents were shown to arise from at least three transitions (26). At present, the transient currents appear to arise from a growing list of membrane proteins that includes such transporters as the Na+/K+ ATPase, the Na+/Ca2+ exchanger, the H'/myo-inositol, the H'/oligopeptide, the Na+/GABA, the Na+/P, and the Na+/glucose cotransporters.
transmembrane helices IV and V in which mutant transporters were analyzed using the X. laevis oocyte expression system. The ability of the mutant transporters to mediate Na\(^+\)/glucose transport was assayed by using the two-electrode voltage clamp, and the sufficiently active mutants were tested for sensitivity to inhibition by the ethylamine and ethylsulfonate MTS\(^1\) derivatives MTSSEA and MTSES, respectively. In previous reports, one of the mutants, A166C has already been characterized in detail using both the X. laevis oocyte system (27) and the COS-7 cell system (28). The results of the present study demonstrate that while none of the residues in this region (residues 162–173) play an absolutely essential role in transport, some are located in close proximity to the pathway that extracellular sodium takes to its binding site in SGLT1. Indeed, the distribution of the cysteine mutants that were sensitive to MTS derivatives suggests that this region forms an α-helix, one surface of which lines a Na\(^+\) pore within SGLT1.

We have also characterized the transient currents exhibited by the single cysteine mutants, F163C, A166C, and L173C, and then extended these studies by combining the mutations to construct multiple cysteine mutants. The results from the double and triple cysteine mutants demonstrate that it is possible to manipulate the membrane potential dependence of the transient currents exhibited by SGLT1 over a range as large as 91 mV. Collectively, our observations represent an important structural localization of a region of SGLT1 that is key in the membrane potential transitions that give rise to the transient currents. We hypothesize that these transitions include the external Na\(^+\) binding event and that the region 162–173 of SGLT1 defined by the various single and multiple cysteine mutants forms the pathway that external Na\(^+\) takes to reach its binding site.

**MATERIALS AND METHODS**

**Molecular Biology**—The multiconing site of the eukaryotic expression vector pMT3 (kindly provided by the Genetics Institute, Boston, MA) was removed by digestion with PstI and KpnI, and the cDNA of rabbit SGLT1 (kindly provided by M. A. Hediger) was subcloned into the remaining EcoRI site. The mutations were introduced into this construct using the megaprimer method of PCR mutagenesis (29) by making mutation-containing PCR products that were digested with BclI and then ligated to BclI-digested pMT3-SGLT1. The mutations and stretch of DNA between the two BclI sites were verified by dideoxy chain termination DNA sequencing. The DNA used for the oocyte injections was prepared using the QIAprep Spin Plasmid Kit (Qiagen, Chatsworth, CA) without further purification. MTSES and MTSEA were obtained commercially (Toronto Research Chemicals, Toronto, Canada).

**Oocyte Preparation**—*X. laevis* frogs were anaesthetized with a 0.17% solution of 3-aminoenobonic acid ethyl ester in water. Stage V or VI oocytes were then surgically removed and digested with 2 mg/ml type I-V collagenase (Sigma) prepared in modified Barth’s saline (MBS) for 60–90 min. The composition of the MBS was 0.88 mM NaCl, 1.0 mM KCl, 2.4 mM NaHCO\(_3\), 15.0 mM HEPES-NaOH (pH 7.6), 0.3 mM Ca(NO\(_3\))\(_2\), 0.41 mM MgCl\(_2\), 0.82 mM CaCl\(_2\), 10 mM MgSO\(_4\), 10 mg/ml penicillin, 10 mg/ml streptomycin. After the collagenase digestion, oocytes were kept in MBS overnight at 18 °C before being injected with the DNA.

**Oocyte Injection**—Using a Drummond Nanoject (Drummond Scientific, Broomall, PA), 4.7 nl of TE buffer (10 mM Tris-HCl, 2 mM EDTA, pH 8) containing 0.15 ng of mutant SGLT1 in pMT3 and 0.15 ng of secreted alkaline phosphatase in pMT3 was injected into the animal pole of the defolliculated oocytes as described previously by Swiek et al. (30). The injected oocytes were kept in MBS supplemented with 2.5 mM sodium pyruvate for 2–3 days before being transferred to 96-well plates to be incubated individually another 16–24 h. The incubation solution from each oocyte was then tested for alkaline phosphatase activity following the protocol of Tate et al. (31). Oocytes that were positive according to this assay were then selected for the electrophysiology, which was conducted over the next 2 days.

**Two-electrode Voltage Clamp**—In all experiments, the oocyte currents were measured with the two-electrode voltage clamp techniques (32) used an Axoclamp-2A amplifier, TL-2 data acquisition system, and pCLAMP software (Axon Instruments, Foster City, CA) to generate voltage pulses and measure the current responses. Oocytes with resting membrane potentials less negative than −30 mV were discarded. Microelectrode resistances ranged from 0.5 to 2.0 megohms. During an experiment, the voltage-clamped oocyte was under the constant perfusion of buffer at approximately 2 ml/min. The composition of the uptake buffer was 100 mM NaCl, 2 mM KCl, 2 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM HEPES-Tris base (pH 7.4). Current responses to 100 ms voltage pulses were recorded at a sample rate of 2.5 ms\(^{-1}\) as the average of the responses to three consecutive trials and were subjected to a 500-kHz, five-point Gaussian filter prior to curve fitting or the calculation of steady state parameters.

**Transient Current Measurements**—In an oocyte expressing SGLT1, the total transient current response following a voltage pulse consists of 1) a nonspecific component and 2) an SGLT1-specific component that is inhibited by phlorizin. In all of our experiments, we isolated the SGLT1-specific transient currents by performing a point by point subtraction of the current response measured immediately before and after the addition of 0.2 mM phlorizin. The principle on which this analysis is based is that nonspecific currents, due mostly to oocyte membrane capacitance, is approximated by the total transient current response measured in the presence of a saturating concentration of phlorizin.

Once the SGLT1-specific transient currents were isolated using the phlorizin subtraction method described above, to obtain the \(Q(V_t)\) relationship, we first base line-corrected and then integrated the transient currents with respect to time. Typically, the transient currents were measured for a series of 100-ms test potentials \(V_t\) ranging from −150 to +50 mV from a holding potential of −50 mV, and the base-line correction was done by subtracting the average values for the currents measured toward the end of these voltage pulses (between 95 and 99 ms). The integration of the SGLT1-specific transients that followed was always done over the entire duration of the voltage pulses (from 0 to 99 ms), and the \(Q(V_t)\) relationships thus obtained were fitted to the Boltzmann distribution,

\[
Q(V_t) = \frac{Q_{\text{max}}}{1 + \exp \left( V_t - V_{1/2} \right)/kT) - Q_{\text{min}} \quad \text{(Eq. 1)}
\]

where \(Q(V_t)\) equals the charge that has moved in response to a voltage jump from the hyperpolarizing limit to \(V_t\). \(V_{1/2}\) corresponds to the maximal charge transfer, \(Q_{\text{max}}\) is \(Q\) at the hyperpolarizing limit, \(V_{1/2}\) is the voltage at which the charge movements are half-complete, \(k\) is an apparent valence, \(F\) is Faraday’s constant, \(R\) is the gas constant, and \(T\) is temperature. Curve fitting was done using the Levenberg Marquardt algorithm (Origin 4.0, Microcal Software, Northampton, MA).

**RESULTS**

**Construction and Verification of the Mutants**—Each amino acid in the region between putative transmembrane helices IV and V, residues 162–173, was replaced individually with cysteine in wild type (WT) rabbit SGLT1 without prior removal of any of the 15 endogenous cysteines. All mutations were verified by DNA sequencing, and except for the desired base changes, the sequence for all of the mutants between and including the BclI restriction enzyme sites was identical to WT. Fig. 1 is a schematic representation of SGLT1 showing the location of the single cysteine mutants. The schematic is based on a proposed secondary structure in which SGLT1 is composed of a short extracellular N terminus followed by 14 α-helical hydrophobic domains that traverse the membrane in zig-zag fashion (33).

\[\text{α-Methyl Glucoside (αMG)-induced Na}^{+}\text{ Currents Mediated by Mutants—The ability of the mutants to function as Na}^{+}/\text{glucose cotransporters was assessed using the X. laevis oocyte expression system and the two-electrode voltage clamp technique. The assay used to screen the mutants for function was a straightforward measurement of the currents induced by increasing concentrations of αMG, a nonmetabolizable SGLT1 sugar substrate. In Fig. 2A, we show the results of a typical experiment with an oocyte expressing the L173C mutant. Here, the αMG concentration was systematically titrated from 0.025\]
were sensitive to inhibition by either MTSES or MTSEA (see "Results").

mutants created in the loop connecting putative transmembrane helices IV and V. Highlighted are the cysteine mutants that were sensitive to inhibition by either MTSES or MTSEA (see "Results").

**TABLE I**

| Mutant | $\alpha$MG apparent $K_m$ (in mM) at $-50$ mV | Mutant | $\alpha$MG apparent $K_m$ (in mM) at $-50$ mV |
|--------|---------------------------------|--------|---------------------------------|
| I162C  | $<0.2$                          | F168C  | $0.055 \pm 0.004$               |
| F163C  | $0.580 \pm 0.038$               | L171C  | $0.070 \pm 0.016$               |
| S164C  | $<0.2$                          | Q170C  | $0.110 \pm 0.013$               |
| G165C  | $<0.2$                          | L173C  | $0.126 \pm 0.012$               |
| A166C  | $0.905 \pm 0.057$               | T172C  | $0.35 \pm 0.041$                |
| I167C  | $0.150 \pm 0.011$               |        |                                 |

**FIG. 1.** Schematic representation of the predicted topology of rabbit SGLT1 (33) showing the location of the single cysteine mutants created in the loop connecting putative transmembrane helices IV and V. Highlighted are the cysteine mutants that were sensitive to inhibition by either MTSES or MTSEA (see "Results").

**FIG. 2.** A, steady state currents induced by increasing concentrations of $\alpha$MG in an oocyte expressing the L173C mutant over a range of membrane potentials (V = $-150$ mV to $-10$ mV, increments of 20 mV). The data are fit to the Michaelis-Menten relationship. B, voltage dependence of the $\alpha$MG apparent $K_m$ obtained for the F163C, A166C, Q170C, and L173C compared with WT SGLT1. The error bars represent S.D. values ($n \geq 3$).

to 1.0 mM, and at each concentration, the currents were measured as the membrane potential was stepped from $-10$ to $-150$ mV in increments of 20 mV. We show that these eight sets of data can be independently fitted to the Michaelis-Menten relationship, $I = I_{max} \frac{[\alpha$MG]}{K_m + [\alpha$MG]}$. This illustrates that when the functional expression is sufficiently high, the steady state $\alpha$MG-induced currents can be used to calculate an apparent $\alpha$MG $K_m$ for the mutant transporter.

From the kind of experiment described above, accurate determinations of an apparent $\alpha$MG $K_m$ were made for the following eight mutants: F163C, A166C, I167C, F168C, Q170C, L171C, T172C, and L173C. Table I summarizes the apparent $\alpha$MG $K_m$ for these cysteine mutants when $V$ = $-50$ mV. For three of the mutants (F163C, A166C, and T172C), the apparent affinities for $\alpha$MG are significantly reduced compared with WT; for another three mutants (I167C, Q170C, and L173C), the affinities are relatively unchanged; and for another two mutants (F168C and L171C), the affinities are higher compared with WT. The remaining four mutants (I162C, S164C, G165C, I169C) exhibited measurable $\alpha$MG-induced currents, but the levels of these currents were too small to allow for accurate determination of an apparent $\alpha$MG $K_m$. From titrations of $\alpha$MG concentrations up to 10 mM $\alpha$MG, the best that could be accomplished for these four mutants was an estimate of an upper bound to the apparent $\alpha$MG affinity (<0.2 mM).

In Fig. 2B we show the voltage dependence of the apparent $\alpha$MG $K_m$ for several of the cysteine mutants. Q170C and L173C exhibit apparent $K_m$ versus $V$ curves that are nearly identical to the one exhibited by WT SGLT1, whereas F163C and A166C demonstrate curves shifted significantly toward reduced apparent $\alpha$MG affinities. In every case, the curves are invariant with respect to membrane potential at sufficiently negative membrane potentials, indicating that for these cysteine mutants the sugar binding step is membrane potential-independent.

The low levels of $\alpha$MG-induced currents exhibited by I162C, S164C, G165C, and I169C can be attributed to low levels of expression caused by the particular cysteine substitution. The possibility that the low current levels were due to poorly performed cDNA injections and/or unhealthy oocytes was ruled out for the following reasons: 1) the low levels of $\alpha$MG-induced currents for oocytes injected with the four mutants were consistently low despite the fact that oocytes from the same batch injected with WT or another mutant cDNA demonstrated much larger currents, and 2) coinjection of cDNA encoding secreted alkaline phosphatase and the secreted alkaline phosphatase assay performed on the same or previous day demonstrated that the oocytes expressing poor $\alpha$MG-induced currents at the same time were expressing high levels of secreted alkaline phosphatase. The low levels of $\alpha$MG-induced currents are therefore probably a direct consequence of the cysteine mutation.

Since the low levels of $\alpha$MG-induced currents were accompanied by reduced amounts of charge movements (see "Charge Movements"), this further suggests that the four mutants I162C, S164C, G165C, and I169C are each expressing poor transport function, probably because they are not being properly trafficked to the plasma membrane. Such an interpretation is consistent with previous reports that charge movements
of WT SGLT1 are correlated to plasma membrane expression levels (32) and that many single amino acid substitutions elsewhere in the SGLT1 sequence do in fact lead to a trafficking defect in which the mutant protein is made at normal levels but poorly processed or never processed to the plasma membrane (10).

Charge Movements—Wild type SGLT1 exhibits transient currents that are capacitive-like charge movements that occur in response to rapid changes in membrane potential. The integral of these transient currents represents a charge movement associated with a conformational change that moves protein charges or dipoles across the membrane electric field or 2) ion movements from the extracellular medium to a binding site within the membrane electric field.

As a secondary assay for screening the function of the various cysteine mutants, we measured the charge movements specific to the mutant transporters by voltage jump experiments in the absence and presence of saturating phlorizin (see “Materials and Methods”) and then fit the \( Q(V) \) relationships thus obtained to the Boltzmann distribution. Table II summarizes the \( V_{0.5} \) determined for 8 of the 12 cysteine mutants. We see that for F163C, A166C, F168C, and L173C mutants expressed in \( X. laevis \) oocytes, the \( V_{0.5} \) is shifted toward more positive potentials and that for Q170C and L171C, the \( V_{0.5} \) is shifted toward more negative potentials. In general terms, shifts in the \( Q(V) \) relationship along the voltage axis are probably due to changes in one or more membrane potential transitions in the SGLT1 transport cycle. These transitions may be associated with the binding of extracellular Na\(^+\) and/or the reorientation of the empty transporter from inside facing to outside facing. The results indicate that the region where the cysteine substitutions were made (amino acids 162–173) has an important role to play in such membrane potential–dependent transitions.

We note that, as with the \( \alpha \)MG-induced currents, the low expression levels precluded an accurate determination of the \( V_{0.5} \) for the remaining four mutants, I162C, I169C, G165C, and S164C, although phlorizin-inhibitable transient currents were clearly detectable in every case.

Sensitivity to MTSEA and/or MTSES Inhibition—For the eight cysteine mutants that demonstrated significant \( \alpha \)MG-induced currents, we also tested whether introduction of the cysteine would allow the sulfhydryl-reactive compound MTSEA or MTSES to inhibit transporter function. Neither compound had any effect on either the steady-state transport kinetics or transient currents exhibited by WT SGLT1, and the experiments were carried out in anticipation that some of the cysteines had been introduced into positions that were not only accessible to the MTS reagents but also in close enough proximity to functionally critical residues that their own chemical modification would lead to an alteration in transporter func-

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**TABLE II**

| Mutant   | \( V_{0.5} \) | Mutant   | \( V_{0.5} \) |
|----------|---------------|----------|---------------|
| I162C    | ND\(^a\)      | F168C    | 24.1 ± 4.41   |
| F163C    | 9.4 ± 0.6     | I169C    | ND            |
| S164C    | ND            | Q170C    | −11.7 ± 1.24  |
| G165C    | ND            | L171C    | −13.3 ± 2.2   |
| A166C    | 19.5 ± 0.76   | T172C    | 3.05 ± 1.55   |
| I167C    | 2.71 ± 3.40   | L173C    | 23.6 ± 3.20   |

\(^a\) ND, not determined.

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![Graph](https://via.placeholder.com/150)

**Fig. 3. A**, time course of the MTSEA inhibition of the F163C, A166C, and L173C mutants expressed in X. laevis oocytes. Shown are the currents induced by \( \alpha \)MG (AMG) at \( V = −50 \) mV. At the time indicated, 1 mM MTSEA was added to the perfusate. \( [\alpha \)MG] = 1 mM for the F163C and A166C data; \([\alpha \)MG] = 0.1 mM for the L173C data. The currents are normalized to the currents induced by \( \alpha \)MG at the start of the time course experiment. The time course of the MTSES inhibition of currents mediated by Q170C. Representative experiment showing the currents induced by 1 mM AMG in an oocyte expressing Q170C. At the time indicated, 1 mM MTSES was added to the buffer perfusing the oocyte. The currents are normalized to the currents induced by 1 mM AMG at the start of the time-course experiment.

The functional screen consisted of first adding \( \alpha \)MG to the buffer perfusing the oocyte and then, while measuring the \( \alpha \)MG-induced currents, adding either 1 mM MTSEA or MTSES to the buffer. For WT, I167C, L171C, and T172C, there was no obvious inhibition of the \( \alpha \)MG-induced currents by either MTS reagent; for F163C, A166C, and L173C there was a dramatic and rapid inhibition of the currents by 1 mM MTSEA but not 1 mM MTSES; for Q170C, there was equally dramatic and rapid inhibition with 1 mM MTSES but not 1 mM MTSEA. In Fig. 3A, the time course of inhibition of currents mediated by F163C, A166C, and L173C by MTSEA is shown, illustrating that the reaction with MTSEA is complete within 1–2 min. The MTSES inhibition time course for Q170C-mediated currents is qualitatively very similar (Fig. 3B).
4 reveals that the αMG-induced Na\(^+\) currents mediated by F163C and A166C are reduced substantially by MTSEA. It is important to note that the inhibition when expressed as a percentage of the αMG-induced Na\(^+\) currents measured prior to MTSEA is not constant with respect to membrane potential. The insets of Fig. 4, show the data replotted as percentage of inhibition by MTSEA as a function of membrane potential from -150 to -30 mV. For each of these two cysteine mutants, the trend is toward a greater percentage of inhibition at more positive membrane potentials. This result is consistent with MTSEA having an effect on the membrane potential dependence of transport. The result is also a simple illustration that the effect of MTSEA cannot be explained solely in terms of a reduction in the number of functional transporters.

**Mutant L173C**—Similar to A166C and F163C, αMG-induced Na\(^+\) currents of L173C are also reduced by MTSEA (see Fig. 5A). But after careful analysis of the MTSEA inhibition of the αMG-induced currents for L173C, it became apparent that the MTSEA effect on L173C was very different from that seen with F163C and A166C. Fig. 5B shows that 1 mM MTSEA inhibits inward currents measured in the absence of sugar in an oocyte expressing L173C. This was never observed for oocytes expressing WT, F163C, or A166C. Occasionally, MTSEA did effect the currents for these oocytes; however, the effect was small (<15 nA) and always in the opposite direction toward greater inward currents. Moreover, the effect with the L173C mutant was irreversible, whereas with WT SGLT1 or the other cysteine mutants the base-line shift was reversed upon wash-out of the MTSEA. It was determined that most of what had appeared initially to be an inhibition of the αMG-induced currents of L173C is in reality due to a shifting of the base line caused by MTSEA exposure.

The explanation for why MTSEA inhibits inward currents mediated by L173C in the absence of αMG may be related to the fact that the L173C mutant exhibits a much larger Na\(^+\) leak compared with WT, F163C, and A166C (Fig. 6, upper panel). This Na\(^+\) leak is experimentally defined as the phlorizin-inhibitable current measured in the absence of sugar substrate and has been interpreted as reflecting uncoupled transport. For the L173C mutant, a significant consequence of MTSEA exposure is a reduction in the rate of uncoupled transport. In contrast, the Na\(^+\) leak currents mediated by WT, F163C, and A166C are not similarly affected by exposure to MTSEA (data not shown).

In addition to the MTSEA effect on the Na\(^+\) leak, is there any other effect on the steady state or kinetics of the L173C mutant? With respect to the αMG-induced steady state currents, there are no significant changes. The apparent αMG \(K_m\) after
MTSEA exposure is 0.111 ± 0.009 mM compared with 0.126 ± 0.012 mM. The $I_{\text{max}}$ following MTSEA exposure also appears unchanged when experimental errors are taken into account.

**MTSEA Affects the Transient Currents of Single Cysteine Mutants F163C, A166C, and L173C**—We measured the effect of MTSEA on the transient currents exhibited by A166C, F163C, and L173C. Fig. 7 shows the normalized $Q(V_t)$ curves for F163C before and after inhibition by 1 mM MTSEA, in which the data represent the pooling of experiments from six different *X. laevis* oocytes expressing F163C. Fig. 7 shows that MTSEA exposure causes a shift in the F163C $Q(V_t)$ curve toward more negative membrane potentials. This is demonstrated quantitatively by the midpoint of the fitted Boltzmann distribution $V_{0.5}$ changing from 9.4 ± 0.6 mV to −9.1 ± 0.4 mV. Similar results were obtained for A166C and L173C, and the parameters describing the normalized $Q(V_t)$ curves before and after MTSEA exposure for all three cysteine mutants are summarized in Table III. We note that the errors referred to in Table III are the errors of the fit to the normalized and pooled $Q(V_t)$ data.

All three single cysteine mutants following exposure to MTSEA demonstrate a significant shift in the $Q(V_t)$ curve toward more negative membrane potentials. F163C and A166C demonstrate shifts approximately equal in magnitude, whereas L173C exhibits a smaller shift. Interestingly, this seems to correlate with the relatively smaller percentage of inhibition of the steady-state currents mediated by L173C by MTSEA. In terms of the total amount of charge transferred from the hyperpolarizing limit to the depolarizing limit, the effect of MTSEA is minimal for all three mutants. The $Q_{\text{max}}$ following MTSEA exposure tends to be within 5–15% of the value measured immediately prior to the exposure (data not shown).

**Double and Triple Cysteine Mutants**—In order to examine further the nature of the MTSEA effect, the three single cysteine mutants described above were combined to produce three double cysteine mutants and a triple cysteine mutant. Specifically, we wanted to determine whether the various permutations and combinations of cysteines at positions 163, 166, and 173, would lead to an enhancement of the MTSEA effect on the transient currents. Two of the double mutants, F163C/L173C and A166C/L173C, expressed significant levels of aMG-induced sodium currents, and as expected, these currents were inhibited by MTSEA. The aMG apparent affinities measured for the two mutants were 0.54 ± 0.05 mM (F163C/L173C) and 0.81 ± 0.04 mM (A166C/L173C) at −50 mV. Interestingly, these affinities seem to parallel the affinities at −50 mV of the F163C and A166C mutants, which were 0.58 ± 0.04 and 0.91 ± 0.06 mM, respectively. For the remaining double mutant (F163C/A166C) and triple mutant there was not enough functional expression to accurately measure MTSEA inhibition of the aMG-induced sodium currents or determine an aMG apparent affinity, although both mutants did clearly exhibit low levels of aMG-induced sodium currents.

All three double mutants and the triple mutant, however, did express sufficiently to allow determination of the effect of MTSEA on the voltage dependence of the transient currents. In Fig. 8A, we show a representative experiment in which transient currents were measured in an oocyte expressing the double mutant F163C/A166C. Using the phlorizin subtraction pro-
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**Fig. 6.** Upper panel, the Na⁺ leak (nA) currents (currents inhibited by 0.2 mM phlorizin) normalized according to the $Q_{\text{max}}$ is shown for rabbit SGLT1 WT, A166C, and L173C. The Na⁺ leak/$Q_{\text{max}}$ (s⁻¹) curve for the F163C mutant is essentially the same as for A166C and has been omitted for clarity. Lower panel, a representative experiment showing that the Na⁺ leak for the L173C mutant is significantly inhibited by the exposure to 1 mM MTSEA.

Protocol to filter out the nonspecific transient currents, we present the SGLT1-specific transient currents for a series of voltage pulses between −150 and 90 mV, before and after a 5-min exposure to 1 mM MTSEA (upper and middle parts, respectively). In every case, the voltage pulse was from a holding potential of −50 mV. The current traces show that subsequent to MTSEA there is significantly more charge transferred by hyperpolarizing voltage jumps from the −50 mV. When the transient currents are integrated to obtain $Q$, this change is clearly seen as a shifting of the $Q(V)$ curve (lower part). In Fig. 8B, we show transient current data from an experiment for the triple cysteine mutant, F163C/A166C/L173C. Here the MTSEA effect is even more pronounced, with larger charge movements following hyperpolarizing voltage jumps and a greater shift in the $Q(V)$ curve. We note that the data in Fig. 8, A and B, also illustrate that the MTSEA effect for the double and triple cysteine mutants does not include a significant change in the total amount of charge transferred from the hyperpolarizing to the depolarizing limit.

Fig. 9 shows a summary of the results from experiments looking at the transient currents before and after MTSEA exposure for the triple cysteine mutant and all three of the double cysteine mutants. The $Q(V)$ data for the multiple cysteine mutants were normalized, pooled, and then fitted to the Boltzmann distribution. Two important observations are noted: 1) the progressive introduction of cysteines leads to a progressive shifting of the $Q(V)$ curves to the right of the one exhibited by WT, and 2) compared with the data for the single cysteine mutants, there was a greater net shift toward negative membrane potentials resulting from MTSEA exposure, with the greatest net shift occurring for the triple mutant.

The shift in the $Q(V)$ curves observed for the cysteine mutants following MTSEA exposure is similar to the shift seen when the external Na⁺ concentration is reduced. It has been shown (2) that a reduction in the external Na⁺ concentration from 100 to 10 mM results in the WT SGLT1 $Q(V)$ shifting toward more negative membrane potentials ($\Delta V_{0.5} = 98$ mV). In Fig. 10, we show the comparable data for A166C in which the $Q(V)$ is determined at 100 mM NaCl and 10 mM NaCl. Like WT SGLT1, the $Q(V)$ is shifted toward more negative membrane potentials by 85 mV, while the apparent valence remains essentially unchanged. In addition, the effect of MTSEA in shifting the $Q(V)$ curve seems to be preserved irrespective of external Na⁺ concentration.

**Mutant Q170C**—As described earlier, MTSEA did not inhibit the αMG-induced sodium currents mediated by Q170C, but MTSES, a negatively charged and bulkier MTS derivative, was inhibiting. In order to investigate whether the lack of an MTSEA effect on function was due to an inability to react with the cysteine introduced at position 170, pretreatment experiments were carried out. In experiments where the oocytes expressing Q170C had been pretreated with 1 mM MTSEA, we found that 1 mM MTSES could no longer inhibit transport activity (data not shown). This indicates that MTSEA can react with the cysteine introduced at position 170 but that the ethyl amminium group left there by the reaction has little effect on transporter function.

Apart from the fact that MTSES was inhibiting and MTSEA was not, the inhibition of Q170C was different from the inhibition of the other three mutants in a number of interesting ways. Recall that the exposure of F163C, A166C, and L173C to MTSEA resulted in a shift of the $Q(V)$ curve to more negative potentials, and only a modest decrease (~15%) in the $Q_{\text{max}}$. The exposure to MTSES of Q170C, however, resulted in no statistically significant shift of the $Q(V)$ curve. Instead there was a substantial decrease (~50%) in the $Q_{\text{max}}$ (see Fig. 11). In addition, the apparent αMG $K_m$ was unchanged by reaction with MTSES. Consequently, the decrease in transport activity observed with Q170C was attributable entirely to a change in the $I_{\text{max}}$. Since MTSES caused both $I_{\text{max}}$ and $Q_{\text{max}}$ to decrease by
approximately the same factor, it is reasonable to conclude that the Q170C turnover rate is relatively unaffected by MTSES.

**DISCUSSION**

**Structural Implications**—The sensitivity to either MTSEA or MTSES inhibition introduced by the cysteine substitutions at positions 163, 166, 170, and 173 indicate that these positions probably localize to a functionally important region of SGLT1. Furthermore, since MTSEA and MTSES both are water-soluble and relatively membrane-impermeant, these four positions must be arranged in the SGLT1 folded structure such that they are accessible from the extracellular space. Together with the observation that the four positions are distributed in periodic fashion along the linear amino acid sequence of SGLT1, this indicates that the region analyzed by our cysteine scanning mutagenesis project (amino acids 162–173) may be an α-helix with one face exposed to the extracellular aqueous environment. In Fig. 12, we show an α-helical wheel representation of this region that highlights those cysteine mutants which were inhibited by either MTSEA or MTSES. We note that these cysteine mutants all cluster along one face of the hypothetical helical arrangement.

The region consisting of amino acids 162–173, based on hydrophathy profiles, is highly hydrophobic. We believe that this lends support to the α-helical model and speculate that this region is a part of a transmembrane segment rather than an extracellular connecting loop, as had been previously proposed (33). The experimental basis for originally considering the region as an extracellular loop was the glycosylation of a mutant with an acceptor insertion between positions 169 and 170 reported by Turk et al. (33). This glycosylation mutant, however, was incapable of transport and considering the rather large size of the insertion (42 amino acids), the topology of this mutant may not be representative of the functional SGLT1 topology.

**Anomalous Behavior of Mutant Q170C**—In many ways, the behavior of Q170C was somewhat aberrant compared with the other single cysteine mutants surveyed in the present study. In particular, Q170C was sensitive to inhibition by MTSES (an MTS derivative with a net negative charge) rather than MTSEA (an MTS derivative with a net positive charge). Furthermore, the effect of MTSES exposure on the transient currents exhibited by Q170C was to reduce their magnitude ($Q_{\text{max}}$ decreases by a factor of $-2$) without significantly changing the kinetics or membrane potential dependence. The most plausible interpretation of these results is that substitution of a net negative charge at the 170-position of SGLT1 changes the number of functional transporters rather than the kinetics of transport. The reasons for this are currently under investigation.

**Functional Implications of Steady State Experiments and the Na"^+ Leak**—Cysteine scanning mutagenesis of SGLT1 has yielded substantial new information on structure/function relationships for Na"^+/glucose cotransport. Our results indicate that the SGLT1 apparent affinity for the sugar substrate aMG is extremely sensitive to amino acid substitutions in the region between positions 162 and 173. Of the 12 consecutive residues studied, seven residues, when changed to a cysteine, result in either a substantially higher or lower apparent affinity. Because we are dealing with a Na"^+-coupled sugar transporter, changes in the apparent affinity for aMG do not necessarily imply that changes to the sugar binding site have occurred. In theory, changes in apparent sugar affinity could also arise from alterations in Na"^+ binding or some other transition linked to sugar binding. Considering that a truncation mutant of SGLT1 has shown that the essential structure for glucose binding and translocation is provided by the C-terminal half of the transporter (35, 36) and that amino acids 162–173 are localized to the N-terminal half, we believe that the effect of the cysteine substitutions studied here reflects changes in Na"^+ binding.

Another observation that supports the hypothesis that the region 162–173 is involved in Na"^+ binding pertains to the Na"^+ leak currents exhibited by L173C. In our analysis of L173C, we have shown that this cysteine mutant demonstrates substantially larger leak currents compared with WT SGLT1. In addition, the modification of L173C with MTSEA results in a significant inhibition of these same leak currents. Since the leak currents represent the turnover of the transporter in the absence of sugar, these results provide compelling evidence that the chemistry at position 173 influences Na"^+ binding and transport.

**Functional Implications of Transient Current Experiments**—We have fit the experimentally determined $Q(V_t)$ curves for the various cysteine mutants to the Boltzmann distribution. As others have found with several different WT SGLT1 isoforms (37), our $Q(V_t)$ data are well described by such a distribution. Recalling that the fundamental assumption with the Boltzmann distribution is that it arises out of a two-state system, we derive the following expression for a system where the two states are Na"^+-bound/unbound:

$$V_{0.5} = \frac{RTzF}{\ln(K_{\text{on}}/K_{\text{off}})}$$  \hspace{1cm} (Eq. 2)

where $K_m$ is the Na"^+ affinity constant in the absence of membrane potential. If the apparent valence is invariant, then according to this expression a shift in the $Q(V_t)$ curve along the membrane potential axis can be due to either a Na"^+ concentration change or a change in the Na"^+ affinity,

$$\Delta V_{0.5} = RTzF \ln([\text{Na}^{+}]/[\text{Na}^{+}]/\text{K}_m$$  \hspace{1cm} (Eq. 3)

$$\Delta V_{0.5} = RTzF \ln(K_{\text{on}}/K_{\text{off}})$$  \hspace{1cm} (Eq. 4)

where $[\text{Na}^{+}]_0$ and $[\text{Na}^{+}]_1$ are the Na"^+ concentrations; $K_m$ and $K_m'$ are the Na"^+ affinity constants. Under such a model, the $\Delta V_{0.5}$ data that we have shown for the cysteine mutants sensitive to MTSEA inhibition (F163C, A166C, and L173C) may be interpreted as evidence that the reaction with MTSEA has reduced the Na"^+ affinity. In conjunction with the double and triple cysteine mutant data, it indicates that the greater the number of cysteines and hence ethyl amines introduced by the reaction, the greater the reduction in the Na"^+ affinity.

Although the two-state simplification and Boltzmann distribution explain the changes in the $Q(V_t)$ curves observed with external Na"^+ concentration changes and MTSEA, the transient currents of SGLT1 exhibit other properties that cannot be accounted for by a two-state model. For instance, when the WT transient currents are measured with sufficiently fast voltage

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**Table III**

Parameters of the $Q(V_t)$ curves for mutants F163C, A166C, and L173C before and after MTSEA exposure

| Mutant | $V_{0.5}$ before MTSEA | $V_{0.5}$ after MTSEA | $\Delta V_{0.5}$ before MTSEA | $\Delta V_{0.5}$ after MTSEA |
|--------|------------------------|-----------------------|-------------------------------|-------------------------------|
| F163C  | $9.4 \pm 0.4 (n = 6)$   | $-9.1 \pm 0.4 (n = 6)$ | $-18.5$                      | $0.98 \pm 0.02$              |
| A166C  | $19.5 \pm 1.0 (n = 8)$  | $1.6 \pm 0.8 (n = 3)$  | $-17.9$                      | $1.00 \pm 0.03$              |
| L173C  | $19.5 \pm 0.9 (n = 4)$  | $7.6 \pm 1.0 (n = 4)$  | $-11.5$                      | $1.22 \pm 0.04$              |

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clamp techniques, their decay can be resolved into at least two exponentials (26).

This suggests that the transition that gives rise to the transient currents, in terms of discrete state kinetics, consists of at least two kinetic steps. We note that the transient current data presented in the present study were acquired with a relatively slow voltage clamp where the voltage pulse rise time was non-negligible compared with the transient current decay time constants. As such, we were prevented from carrying out a quantitative analysis of the transient current decay for the double and triple cysteine mutants. Nonetheless, preliminary results from experiments using faster voltage clamp techniques suggest to us that the cysteine mutants, like WT SGLT1, also demonstrate multiexponential transient current decays.

Another observation that requires a multistep kinetic model has to do with how the rate of transient current decay is

\[^2\] B. Lo and M. Silverman, unpublished results.

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**Fig. 8.** A, transient currents for the double cysteine mutant F163C/A166C. Nonspecific transient currents have been removed using the phlorizin subtraction protocol (see "Materials and Methods"). SGLT-specific transient currents for a series of voltage pulses between −50 and 90 mV are shown before (upper part) and after (middle part) a 5-min exposure to 1 mM MTSEA. The lower part shows the results of integrating these transient currents to obtain \(Q\) as a function of \(V_t\). B, transient currents for the triple cysteine mutant F163C/A166C/L173C measured using a similar protocol as in A. Upper part, before exposure to MTSEA; middle part, after exposure to MTSEA. The lower part shows the integrated \(Q(V_t)\) curves before and after MTSEA.
influenced by external Na\(^+\) concentration. It has been shown that the rate of decay increases with a decrease in the external Na\(^+\) concentration (2), which is exactly the opposite of what a two-state single-step transition involving Na\(^+\) binding would predict (22). Therefore, the transition undergone by SGLT1 in response to voltage jumps must be a multistep transition with the following two constraints; the multistep transition must 1) include the Na\(^+\) binding event and 2) be constrained in such a way that the steady-state aspect of the transient currents represented by \(Q(V)\) curves is well approximated by a two-state Boltzmann distribution.

The increase in transient current decay rate seen with a reduction in Na\(^+\) concentration is also seen with the cysteine mutants following MTSEA inhibition. Examination of the double and triple cysteine mutant transient currents in Fig. 8, A and B, shows that the decay rates are qualitatively faster after MTSEA exposure. In addition, we have shown quantitative decay rate increases for the single cysteine mutant A166C (27).

These observations can be interpreted as further evidence that what MTSEA does to the cysteine mutants is effectively reduce the external Na\(^+\) concentration. Along with the \(\Delta V_{0.5}\) data, this supports our hypothesis that the MTSEA reaction with the various cysteine mutants is affecting a transition in the transport cycle that includes the Na\(^+\) binding event.

A globally consistent and detailed kinetic description of the currents measured during a voltage jump experiment requires more complexity than the two-state model we have presented. To account for multiexponential decays and the dependence of the rate of decay on external Na\(^+\) concentration, a comprehensive model should include additional states beyond the Na\(^+\)-bound and -unbound states. At present, simulations of WT SGLT1 transient current data have been carried out independently by two different laboratories (4, 26), yielding three- and four-state models, respectively. We are in the process of analyzing our cysteine mutant and MTSEA data in terms of such models. Unfortunately, there has yet to be an experimental method developed that isolates the individual steps in these multistep models, and thus a microscopic description of the
events that give rise to the transient currents remains elusive. We remain without direct experimental evidence as to whether the transient currents arise from ion binding, protein conformational changes that move side-chain charges or dipoles across the membrane electric field, or a combination of both of these possibilities. The $Q(V_t)$ data for the cysteine mutants before and after MTSEA, therefore, may be affecting either Na$^+$ binding directly or indirectly via a kinetic step that precedes or follows the Na$^+$ binding step. Until the possible existence of such kinetic steps are better defined, the Boltzmann distribution and two-state interpretation remain a useful simplification to the analysis of the $Q(V_t)$ data.

In summary, we have presented data showing that SGLT1 transient currents are significantly affected by MTSEA modification of cysteines that have been introduced into positions 163, 166, and 173 of the transporter. Specifically, the $Q(V_t)$ curves of the cysteine mutants are seen to shift toward more negative membrane potentials following MTSEA reaction. This observed $\Delta V_{0.5}$, together with an increase in the rate of transient current decay, suggests that the MTSEA is having an effect similar to the one seen with a reduction in external Na$^+$ concentration. The implication is that MTSEA is affecting a transition that involves the binding of Na$^+$ to the outside facing transporter. This is in keeping with the earlier suggestion that changes in the $\alpha$MG apparent affinity and Na$^+$ leak seen with some of the cysteine mutants relate to alterations in Na$^+$ binding. This finding, together with the evidence that the region 162–173 forms an $\alpha$-helical structure with one surface exposed to the extracellular space, leads us to speculate that this region constitutes part of an external Na$^+$ pore in the SGLT1 protein.

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REFERENCES
1. Hediger, M. A., Coady, M. J., Ikeda, T. S., and Wright, E. M. (1987) Nature 330, 379–381
2. Hazama, A., Loo, D. D., and Wright, E. M. (1997) J. Membr. Biol. 155, 175–186
3. Panayotova-Heiermann, M., Loo, D. D. F., and Wright, E. M. (1995) J. Biol. Chem. 270, 27089–27105
4. Loo, D. D., Hazama, A., Supplisson, S., Turk, E., and Wright, E. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5767–5771
5. Hirayama, B. A., Loo, M. P., Panayotova-Heiermann, M., Loo, D. D., Turk, E., and Wright, E. M. (1996) Am. J. Physiol. 270, G919–G926
6. Lee, W. S., Kanai, Y., Wells, R. G., and Hediger, M. A. (1994) J. Biol. Chem. 269, 12025–12039
7. Chen, X. Z., Coady, M. J., Jackson, F., Berteloot, A., and Lapointe, J. Y. (1995) Biophys. J. 69, 2405–2414
8. Parenti-Lugi, Supplisson, S., Loo, D. D., and Wright, E. M. (1992) J. Membr. Biol. 125, 49–62
9. Bennett, E., and Kimmich, G. A. (1996) Biophys. J. 70, 1676–1688
10. Wright, E. M., Loo, D. D., Turk, E., and Hirayama, B. A. (1996) Curr. Opin. Cell Biol. 8, 468–473
11. Hediger, M. A., Kanai, Y., You, G., and Nussberger, S. (1995) J. Physiol. (Lond.) 482, 73–175
12. Lauger, P., and Jauch, P. (1986) J. Membr. Biol. 91, 275–284
13. Kimmich, G. A., and Carter-Su, C. (1978) Am. J. Physiol. 235, C73–C81
14. Hilgemann, D. W. (1966) Ann. N. Y. Acad. Sci. 770, 136–158
15. Rakowski, R. F., Gadsby, D. C., and De Weer, P. (1997) J. Membr. Biol. 155, 105–112
16. Hilgemann, D. W., Nicoll, D. A., and Philipson, K. D. (1991) Nature 325, 715–718
17. Klahn, E. M., Drew, M. E., Landfear, S. M., and Kavanaugh, M. P. (1966) J. Biol. Chem. 271, 14937–14943
18. Mackenzie, B., Loo, D. D. F., Fei, Y.-J., Liu, W., Ganapathy, V., Leibach, F. H., and Wright, E. M. (1986) J. Biol. Chem. 271, 5430–5437
19. Nussberger, S., Steel, A., Troiti, D., Romero, M. F., Boron, W. F., and Hediger, M. A. (1997) J. Biol. Chem. 272, 7777–7778
20. Wadiche, J. I., Arriza, J. L., Amara, S. G., and Weer, H. A. (1996) J. Neurosci. 16, 5405–5414
21. Forster, I. C., Wagner, C. A., Busch, A. E., Lang, F., Biber, J., Hernando, N., Murer, H., and Werner, A. (1997) J. Membr. Biol. 160, 9–25
22. Mackenzie, B., Loo, D. D. F., Panayotova-Heiermann, M., and Wright, E. M. (1995) J. Biol. Chem. 271, 32078–32083
23. Parenti-Lugi, Supplisson, S., Loo, D. D., and Wright, E. M. (1992) J. Membr. Biol. 125, 63–79
24. Taghialatela, M., Toro, L., and Stefani, E. (1992) Biophys. J. 61, 78–82
25. Chen, X. Z., Coady, M. J., Jackson, F., Berteloot, A., and Lapointe, J. Y. (1995) J. Biol. Chem. 270, 2544–2552
26. Lo, B., and Silverman, H. M. (1998) J. Biol. Chem. 273, 903–909
27. Vayro, S., Lo, B., and Silverman, H. M. (1998) Biochem. J. 332, 119–125
28. Sarkar, G., and Sommer, S. S. (1990) BioTechniques 8, 494–497
29. Swick, A. G., Janicott, M., Chervul-Kastelic, T., McLenathan, J. C., Lane, M. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1812–1816
30. Tate, S. S., Uraer, R., Micanovic, R., Gerber, L., and Underfriend, S. (1992) FEBS J. 4, 227–231
31. Zampighi, G. A., Kreman, M., Boorer, K. J., Loo, D. D., Bezanilla, F., Chandy, G., Hall, J. E., and Wright, E. M. (1995) J. Membr. Biol. 148, 65–78
32. Turk, E., Kern, C. J., Loo, M. P., and Wright, E. M. (1996) J. Membr. Biol. 271, 1925–1934
33. Deleted in proof
34. Panayotova-Heiermann, M., Loo, D. D. F., Konst, C. T., Lever, J. E., and Wright, E. M. (1996) J. Biol. Chem. 271, 10029–10034
35. Panayotova-Heiermann, M., Eskandari, S., Turk, E., Zampighi, G. A., and Wright, E. M. (1997) J. Biol. Chem. 272, 20324–20327
36. Panayotova-Heiermann, M., Loo, D. D. F., Loo, M. P., and Wright, E. M. (1994) J. Biol. Chem. 269, 21016
37. Panayotova-Heiermann, M., Loo, D. D. F., Lostao, M. P., and Wright, E. M. (1997) J. Biol. Chem. 272, 20324–20327