Replacement of Ala-166 with Cysteine in the High Affinity Rabbit Sodium/Glucose Transporter Alters Transport Kinetics and Allows Methanethiosulfonate Ethylamine to Inhibit Transporter Function*

(Received for publication, July 17, 1997, and in revised form, October 22, 1997)

Bryan Lo‡ and Mel Silverman§

From the Medical Research Council Group in Membrane Biology, Department of Medicine, University of Toronto, Toronto, Ontario M5S 1A8, Canada

An alanine to cysteine mutation at position 166 has been introduced by site-directed mutagenesis into the rabbit sodium/glucose transporter (rSGLT1). When expressed in Xenopus laevis oocytes, this mutant transporter (A166C rSGLT1) demonstrates a significantly lower apparent affinity for α-methyl glucoside (αMG) compared with the wild-type transporter (apparent $K_m = 0.8$ versus $0.15$ mm). Using the two-electrode voltage clamp technique, transient currents have also been measured, and for the mutant transporter, the transients induced by large depolarizations exhibit longer time constants than those for wild type. Moreover, the substitution of Ala-166 with a cysteine allows the sulfhydryl specific reagent, methanethiosulfonate ethylamine (MTSEA), to react with and alter the function of the transporter. Whereas the wild-type transporter is unaffected by reaction with MTSEA, A166C rSGLT1 has its steady-state currents induced by 1 mM αMG inhibited 83% within a minute of exposure to MTSEA. Furthermore, the pre-steady-state transients of the A166C mutant after MTSEA exposure demonstrate much shorter time constants than before while the total amount of charge transferred is only slightly diminished. These results together provide evidence that position 166 is situated in a region critical to the functioning of rSGLT1.

The sodium/glucose cotransporter is one member of a family of sodium-dependent transport proteins found in the brush border membrane of intestinal and kidney epithelia (1). It uses the sodium electrochemical gradient to drive the transport of sugar into epithelial cells and is specifically inhibited by phlorizin. Since cloning in 1987 from a rabbit small intestinal cDNA library (2), the high affinity sodium/glucose transporter (SGLT1) has been extensively characterized. The expression of SGLT1 in Xenopus laevis oocytes has allowed for the application of electrophysiologic techniques to measure in detail the kinetics of the transporter. The two-electrode voltage clamp method and the cut open oocyte technique have been used to measure the sodium currents mediated by the transporter, and from such measurements, substrate affinities as well as the sodium:glucose coupling stoichiometry have been determined (3, 4). In addition, electrophysiology has been used to demonstrate that in the absence of sugar or phlorizin, SGLT1 exhibits pre-steady-state currents in response to rapid changes in membrane potential. These transient currents have been hypothesized to be due to charge movements associated with conformational changes of the transporter, and by studying them, estimates have been made of the rate constants for sodium association/dissociation and the reorientation of the empty transporter from an outside facing to inside facing conformation (5).

A number of studies have combined site-directed mutagenesis with the oocyte expression system to identify residues that contribute to the functioning of SGLT1 and its proper trafficking to the plasma membrane (6, 7). A set of glycosylation mutants has yielded information on topology, and a chimera of the high affinity and low affinity isoforms has suggested that sugar binding is determined by the C-terminal half of the protein (8). From comparisons of SGLT1 homologues cloned from different species, certain residues have been hypothesized to be important in determining the kinetic differences between these isoforms (9). Direct experimental evidence elucidating specific structure/function relationships is, however, lacking, and the exact residues that make up the substrate binding sites or determine the coupling of sodium binding to glucose transport remain unknown.

In this study, we report on the characterization of a single cysteine mutant of rSGLT1 with properties distinct from wild type that was identified during a cysteine scanning mutagenesis project. The alanine at position 166, which was replaced with cysteine to generate this mutant, had not previously been hypothesized to be important, although in one glucose galactosetose malabsorption patient, it was found mutated to a threonine (10). Ala-166 is conserved across the rabbit/rat/human isoforms of SGLT1 as well as SGLT2 and the sodium myoinositol transporter. This is not surprising given the high degree of sequence identity for these members of the sodium cotransporter family. What was unexpected was the degree to which some of the steady-state and pre-steady-state kinetics of the transporter was changed by the relatively conservative alanine to cysteine substitution. To date, there have been no other single-site mutations reported that produce dramatic changes to both the steady-state and transient currents mediated by SGLT1.

In addition to the kinetic changes, we also observed that the A166C mutation introduced sensitivity to the cysteine-specific reagent methanethiosulfonate ethylamine (MTSEA). Belonging to a class of compounds known as MTS derivatives, MTSEA...
has been used in combination with cysteine site-directed mutagenesis in the study of proteins such as ACh receptor channel, GABA receptor channel, lactose permease, and voltage-gated ion channels (11–14). We present here experimental evidence that such an approach can also be taken with SGLT1, providing unique insight into certain structure/function relationships for this transporter.

**MATERIALS AND METHODS**

**Molecular Biology—**The multicloning 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 rSGLT1 (kindly provided by M. Heidiger) was subcloned into the remaining EcoRI site. The A166C mutation was introduced into this construct using the megaprimer method of polymerase chain reaction mutagenesis (15) with 5'-TCGGAGCTCTCTTCGTTTG-3' as the sense primer, 5'-TACCCAGAAGATGAGCAGGGCGTCCTT-3' as the antisense primer, and 5'-GGATGAGATGATCCGAAAAGATG-3' as the mutagenic primer. These primers were used to make a mutation-containing polymerase chain reaction product that was digested with BclI and then ligated to BclI-digested pMT3-rSGLT1 using a cycle ligation protocol (16). The mutation and the stretch of DNA between the two BclI sites was verified by dideoxy chain termination DNA sequencing using the Pharmacia Biotech Inc. T7 polymerase sequencing kit. The DNA used for the oocyte injections was prepared using the QIAprep Spin Plasmid Kit (Qiagen, Chatsworth, CA) without further purification.

**Oocyte Preparation and Injection—**X. laevis frogs were anesthetized with a 0.17% solution of 3-aminobenzoic acid ethyl ester in water. Stage V or VI oocytes were then surgically removed and digested with 2 mg/ml of type IV 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 NaHCO3, 15.0 mM HEPES-NaOH (pH 7.6), 0.3 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 10 mg/ml penicillin, and 10 mg/ml streptomycin. After the collagenase digestion, oocytes were kept in MBS overnight at 18 °C before being injected with the DNA.

Using a Drummond Nanojet (Drummond Scientific, Broomall, PA), 4.7 nl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) containing 0.15 ng of pMT3-rSGLT1 or pMT3-A166C-rSGLT1 and 0.15 ng of pMT3-SEAP was injected into the animal pole of the defolliculated oocytes as described previously by Swick et al. (17). The injected oocytes were kept in MBS supplemented with 2.5 mM sodium pyruvate for 2–3 days before transfer to 96-well plates and incubation 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. (18). Oocytes that were positive according to this assay were then selected for the electrophysiology that was conducted over the next 2 days.

**Two-electrode Voltage Clamp—**In all experiments, the oocyte current responses were measured with the two-electrode voltage clamp technique (19). We 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. During an experiment, the voltage clamped oocyte was under the constant perfusion of buffer at approximately 2 ml/min. The composition of this buffer was 100 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1 mM CaCl2, and 10 mM HEPES-Tris base (pH 7.4). Current responses 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 Hz, 5 point gaussian filter prior to curve fitting or the calculation of steady-state parameters. Curve fitting was done either using the Simplix method with the pCLAMP software or using the Levenberg Marquardt algorithm (Origin 4.0, Microcal Software, Northampton, MA). Time constants describing the transient currents were obtained only from data collected in which the voltage clamp was sufficiently fast. Specifically, by fitting the current responses collected in the presence of 0.2 mM phlorizin to a single exponential, the time constant of the voltage clamp for these experiments was estimated to be between 0.4 and 0.7 ms.

**RESULTS**

Fig. 1 shows the current responses to a series of 100-ms voltage steps (−150 to 90 mV in 20-mV increments) from a −50 mV holding potential before (panel A) and after (panel B) the addition of 10 mM αMG for an oocyte expressing A166C rSGLT1. As described previously for wild-type SGLT1 (3), the presence of αMG in the bath solution induces an inward sodium current that is not observed in water-injected oocytes. Moreover, A166C rSGLT1 expression results in current responses which consist of a capacitative transient followed by a slow decay to steady state (most clearly seen for voltage steps to positive potentials). The slow decay has been extensively characterized in wild-type SGLT1 as a pre-steady-state current associated with sodium binding and the reorientation of the empty carrier (5). As with wild-type SGLT1, this pre-steady state current is abolished by the addition of αMG. Fig. 1C shows, for the same representative oocyte expressing A166C, the I-V relationship of the current induced by the 10 mM αMG and the I-V relationship of the sodium leak. In this and all other experiments, the αMG-induced current is defined as the calculated difference in a measurement made in the absence of αMG and a second measurement made immediately afterward in the presence of αMG; the sodium leak is defined experimentally as the current inhibited by saturating phlorizin concentrations in the absence of αMG. In Fig. 1D, we compare the sodium leak of the A166C mutant and wild-type SGLT1 by normalizing with respect to Imax (Fig. 2A). Clearly, the A166C...
Ala-166 Cysteine Mutation of SGLT1

Fig. 2. A, sugar-induced sodium currents as a function of sugar concentration for A166C rSGLT1. In this representative experiment, an oocyte expressing A166C rSGLT1 was voltage clamped and exposed to a bath solution containing 100 mM sodium and a varying amount of MG (0.1, 0.5, 1.0, 2.0, 5.0, and 10.0 mM). Current responses were recorded before and after the addition of each concentration of MG for a series of 100-ms voltage steps. The steady-state current (i.e., the currents recorded during the final few milliseconds of the voltage pulse) induced by the MG was taken as the difference between these two measurements. These MG-induced currents as a function of MG concentration are shown here fitted to the Michaelis-Menton relationship as described for A166C rSGLT1. The data from a series of I-V curves for A166C rSGLT1 are replotted as functions of MG concentrations in Fig. 2A and fitted to the Michaelis-Menten equation to obtain values for an apparent K_M. The results from fitting data from a number of different oocytes expressing either A166C rSGLT1 or wt SGLT1 are presented in Fig. 2B. Clearly, the apparent affinity of MG has been lowered by the alanine to cysteine substitution. In experiments where the MG-induced currents were determined for a series of sodium concentrations ranging from 0 to 100 mM, data were obtained which showed that the mutation did not appear to affect stoichiometry or the apparent sodium affinity. In Fig. 2C, a representative experiment is shown in which the sodium dependence of a 1 mM MG-induced current at −30, −50, and −70 mV is fitted to the Hill equation.

An interesting consequence of the introduction of the cysteine at position 166 was the effect of allowing MTSEA to react with the transporter and alter its function. In Fig. 3, the time course of inhibition in the absence of MTSEA exposure under such conditions resulted in the mutant exhibiting a much smaller sodium leak at negative membrane potentials.

Since the MG-induced currents for A166C rSGLT1 were, generally speaking, comparable in magnitude with those observed for wt SGLT1, we were able to proceed with a characterization of the steady-state transport kinetics of the mutant. The data from a series of I-V curves for A166C rSGLT1 are shown to be inhibited by the addition of 1 mM MTSEA to the bath solution. The inhibition by the MTSEA exhibited the same rapid kinetics in the presence of saturating concentrations of sodium as at lower concentrations. Although we could not directly monitor the time course of inhibition in the absence of MTSEA exposure under such conditions resulted in the

made at a particular sodium concentration before and after the addition of 1 mM MG. The data are fitted to the Hill equation, I = I_max [Na]^(n[H] + K_n), where I is current, I_max is the maximal current at saturating [Na], K_n is the [Na] at which I = I_max/2 (i.e., apparent sodium affinity), and n is the Hill coefficient. For −70 mV, the curve fitting gives I_max = −134 ± 6 nA, K_n = 19.9 ± 2.0 mM, n = 1.32 ± 0.1; for −50 mV, I_max = −115 ± 6 nA, K_n = 24.2 ± 2.2 mM, n = 1.66 ± 0.15; for −30 mV, I_max = −87 ± 4 nA, K_n = 28.2 ± 2.1 mM, n = 2.5 ± 0.3; errors are the errors of the fit.
same degree of inhibition. Moreover, saturating concentrations of the competitive inhibitor phlorizin had no effect on the degree of the MTSEA inhibition nor did replacing the 100 mM NaCl with 100 mM choline-Cl (data not shown).

The inhibition by the MTSEA did not eliminate the αMG-induced currents although the kinetics of the curve in Fig. 3 demonstrates that the effect of MTSEA is both on the degree of the MTSEA inhibition nor did replacing the 100 mM NaCl with 100 mM choline-Cl (data not shown).

The inhibition by the MTSEA did not eliminate the αMG-induced currents although the kinetics of the curve in Fig. 3 indicate that the reaction has gone to completion. Analysis of the αMG currents remaining after exposure to MTSEA (Fig. 4) demonstrates that the effect of MTSEA is both on the degree of the MTSEA inhibition nor did replacing the 100 mM NaCl with 100 mM choline-Cl (data not shown).

The inhibition by the MTSEA did not eliminate the αMG-induced currents although the kinetics of the curve in Fig. 3 indicate that the reaction has gone to completion. Analysis of the αMG currents remaining after exposure to MTSEA (Fig. 4) demonstrates that the effect of MTSEA is both on the degree of the MTSEA inhibition nor did replacing the 100 mM NaCl with 100 mM choline-Cl (data not shown).

The inhibition by the MTSEA did not eliminate the αMG-induced currents although the kinetics of the curve in Fig. 3 indicate that the reaction has gone to completion. Analysis of the αMG currents remaining after exposure to MTSEA (Fig. 4) demonstrates that the effect of MTSEA is both on the degree of the MTSEA inhibition nor did replacing the 100 mM NaCl with 100 mM choline-Cl (data not shown).

The inhibition by the MTSEA did not eliminate the αMG-induced currents although the kinetics of the curve in Fig. 3 indicate that the reaction has gone to completion. Analysis of the αMG currents remaining after exposure to MTSEA (Fig. 4) demonstrates that the effect of MTSEA is both on the degree of the MTSEA inhibition nor did replacing the 100 mM NaCl with 100 mM choline-Cl (data not shown).

The inhibition by the MTSEA did not eliminate the αMG-induced currents although the kinetics of the curve in Fig. 3 indicate that the reaction has gone to completion. Analysis of the αMG currents remaining after exposure to MTSEA (Fig. 4) demonstrates that the effect of MTSEA is both on the degree of the MTSEA inhibition nor did replacing the 100 mM NaCl with 100 mM choline-Cl (data not shown).

The inhibition by the MTSEA did not eliminate the αMG-induced currents although the kinetics of the curve in Fig. 3 indicate that the reaction has gone to completion. Analysis of the αMG currents remaining after exposure to MTSEA (Fig. 4) demonstrates that the effect of MTSEA is both on the degree of the MTSEA inhibition nor did replacing the 100 mM NaCl with 100 mM choline-Cl (data not shown).

The inhibition by the MTSEA did not eliminate the αMG-induced currents although the kinetics of the curve in Fig. 3 indicate that the reaction has gone to completion. Analysis of the αMG currents remaining after exposure to MTSEA (Fig. 4) demonstrates that the effect of MTSEA is both on the degree of the MTSEA inhibition nor did replacing the 100 mM NaCl with 100 mM choline-Cl (data not shown).

The inhibition by the MTSEA did not eliminate the αMG-induced currents although the kinetics of the curve in Fig. 3 indicate that the reaction has gone to completion. Analysis of the αMG currents remaining after exposure to MTSEA (Fig. 4) demonstrates that the effect of MTSEA is both on the degree of the MTSEA inhibition nor did replacing the 100 mM NaCl with 100 mM choline-Cl (data not shown).

The inhibition by the MTSEA did not eliminate the αMG-induced currents although the kinetics of the curve in Fig. 3 indicate that the reaction has gone to completion. Analysis of the αMG currents remaining after exposure to MTSEA (Fig. 4) demonstrates that the effect of MTSEA is both on the degree of the MTSEA inhibition nor did replacing the 100 mM NaCl with 100 mM choline-Cl (data not shown).
Fig. 6. A charge transfer $Q$ as a function of the test potential $V_t$ for A166C before and after MTSEA inhibition. Data are shown for a representative oocyte expressing the A166C before and after a 5-min exposure to 1 mM MTSEA. $Q$ was calculated by integrating the phlorizin-sensitive current responses to 100-ms voltage pulses $V_t$. The holding potential $V_h$ was −50 mV, and prior to integration, the current transients were baseline corrected using the mean current values obtained over the final few milliseconds of the voltage pulse. The data are fitted to the Boltzmann relation, $Q = Q_{\text{max}}/[1 + \exp(V_t - V_{0.5})/kF/RT] - Q_{\text{hyp}}$, where $Q_{\text{max}}$ is the maximal charge transfer, $Q_{\text{hyp}}$ is $Q$ at the hyperpolarizing limit, $z$ is apparent valence, $F$ is Faraday’s constant, $R$ is the gas constant, and $T$ is temperature. For A166C before MTSEA exposure, $Q_{\text{max}} = 37.37\pm 468$ pC, $V_{0.5} = 17.44\pm 0.91$ mV, $z = 1.01\pm 0.03$, $Q_{\text{hyp}} = 27.59\pm 158$ pC. For A166C after MTSEA exposure, $Q_{\text{max}} = 35.97\pm 355$ pC, $V_{0.5} = 8.14\pm 0.79$ mV, $z = 1.03\pm 0.03$, $Q_{\text{hyp}} = 52.63\pm 174$ pC. B, normalized charge transfer $Q_{\text{norm}} = (Q - Q_{\text{hyp}})/Q_{\text{max}}$ as a function of $V_t$. Data are shown for the mean of three experiments for wtSGLT1, eight experiments for A166C, and three experiments for A166C after MTSEA inhibition. Error bars represent the standard deviations. Superimposed on the data are the corresponding Boltzmann relation curves; for wt, $V_{0.5} = -2.54\pm 0.70$ mV, $z = 1.00\pm 0.03$; for A166C, $V_{0.5} = 19.53\pm 0.95$ mV, $z = 1.00\pm 0.03$; for A166C after MTSEA, $V_{0.5} = 1.59\pm 0.78$ mV, $z = 0.99\pm 0.03$.

MTSES pretreatment protects against MTSEA inhibition. A representative experiment in which four oocytes harvested from the same frog and expressing A166C were subjected to 10 mM MTSES for varying amounts of time. The MTSES was removed, and then 1 mM cMG was applied to the bath followed by 1 mM MTSEA. The graph shows the calculated percent inhibition by the MTSEA of the currents induced by the 1 mM cMG. Each of the four oocytes exhibited approximately the same current levels induced by 1 mM cMG before treatment with the MTSEA.

Fig. 7. MTSES pretreatment protects against MTSEA inhibition. A representative experiment in which four oocytes harvested from the same frog and expressing A166C were subjected to 10 mM MTSES for varying amounts of time. The MTSES was removed, and then 1 mM cMG was applied to the bath followed by 1 mM MTSEA. The graph shows the calculated percent inhibition by the MTSEA of the currents induced by the 1 mM cMG. Each of the four oocytes exhibited approximately the same current levels induced by 1 mM cMG before treatment with the MTSEA.

Fig. 7. MTSES pretreatment protects against MTSEA inhibition. A representative experiment in which four oocytes harvested from the same frog and expressing A166C were subjected to 10 mM MTSES for varying amounts of time. The MTSES was removed, and then 1 mM cMG was applied to the bath followed by 1 mM MTSEA. The graph shows the calculated percent inhibition by the MTSEA of the currents induced by the 1 mM cMG. Each of the four oocytes exhibited approximately the same current levels induced by 1 mM cMG before treatment with the MTSEA.

DISCUSSION

Based on theoretical considerations and data from glycosylation mutants, position 166 is hypothesized to be part of a short 16 amino acid loop connecting transmembrane helices IV and V of SGLT1 (21). To date, the only experimental data ascribing function to any part of this loop comes from a study by Panayotova-Heiermann, et al. (6) in which the aspartic acid at position 176 (located at the transmembrane V end of the loop) was mutated to an alanine. In this study, the D176A mutation was shown to result in 1) an increased rate of decay for the current transients corresponding to membrane depolarizations and 2) a shift of the $Q$ versus $V_t$ curve along the voltage axis toward more hyperpolarizing potentials. In contrast, we have shown that an A166C mutation results in these pre-steady-state parameters changing in the opposite direction, namely in 1) a decreased rate of decay for these same current transients and 2) a shift of the $Q$ versus $V_t$ curve along the voltage axis toward more depolarizing potentials. Since an aspartic acid to alanine change is from an electronegative to neutral amino acid and an alanine to cysteine change is from a neutral to relatively electronegative amino acid, these results are consistent with...
the hypothesis that this putative extracellular loop is sensitive to changes in charge and/or polarity. In fact, the direction in which the polarity is adjusted seems to determine the direction in which the time constants describing these transient currents ($\tau_{in}$) change and the $Q$ versus $V_t$ curve shifts.

Referring to the modelling of SGLT1 current transients by Loo and Wright (5), the above changes in pre-steady-state parameters caused by the mutations may be interpreted in terms of conformational transitions. These conformational transitions are induced by changes in membrane potential and involve the movement of charged residues and sodium ions through the membrane electric field. For membrane depolarizations, the transition would be the dissociation of sodium followed by reorientation of the unloaded transporter from outside to inside facing (i.e., Na$^+_{out} \rightarrow$ C$_{out} \rightarrow$ C$_{in}$, where C$_{out}$ and C$_{in}$ represent the outside and inside facing conformations of the cotransporter). Therefore, the observation that the A166C mutant exhibits a slower $\tau_{in}$ (for $V_t = +90$ mV) than wild type implies that the cysteine substitution has caused a decrease in the rate of this Na$^+_{out} \rightarrow$ C$_{in}$ transition. Moreover, the change in the $Q/V_t$ relationship may be interpreted as a change in the voltage-sensing capacity of the transporter.

It is useful to apply the same kind of interpretation to the pre-steady-state data relating to the effect of MTSEA on the A166C mutant. Here we have shown that reaction with MTSEA reduces the value for $\tau_{in}$ (for $V_t = +90$ mV) and shifts the $Q$ versus $V_t$ curve along the voltage axis toward more depolarizing potentials such that the normalized $Q$ versus $V_t$ curve is indistinguishable from that of wild type. Assuming that the MTSEA is reacting with the cysteine introduced at 166 and therefore placing an electropositive ethyl amine group at this location, these results are consistent with the notion presented above, namely that the region around 166 is sensitive to changes in charge and/or polarity. Furthermore, they lend support to the idea that this region is important in defining the energetics of the Na$^+_{out} \rightarrow$ C$_{in}$ transition and the transporter’s voltage-sensing capacity.

In addition to alterations in pre-steady-state parameters, the A166C mutation also results in changes in the steady-state operation of the transporter. Most notably, the A166C mutant demonstrates a significantly lower $\alpha$MG apparent affinity, an observation which indicates that the mutation has affected either the true $\alpha$MG affinity or a transition that is directly coupled to sugar binding. We consider two possibilities, 1) that position 166 is located in the sugar binding site and has a direct influence on that event, and 2) that position 166 is in a region that can influence the sodium activation of sugar binding, perhaps sodium binding itself. The detailed characterization of the transient currents exhibited by the A166C mutant, unfortunately, do not distinguish between these two possibilities, although analysis of the MTSEA effect argues against the first one. Since the inhibition by the MTSEA appears to be unaffected by saturating concentrations of $\alpha$MG and phlorizin, the cysteine at 166 reacting with the MTSEA is unlikely to be located within either the $\alpha$MG or the phlorizin binding sites. The decrease in the $\alpha$MG affinity upon substituting alanine 166 for cysteine and the further decrease in affinity upon reaction with MTSEA, we believe, are due to an influence on the ability of sodium binding to activate $\alpha$MG binding and then transport.

Another piece of evidence that supports the hypothesis that 166 is located in a region important to sodium binding and the coupling of this event to sugar transport is the order of magnitude lower sodium leak exhibited by the A166C mutant as compared with that for wild type. The comparison holds for the case where the leak was normalized to $I_{max}$ and also for the case where the leak was normalized to $Q_{max}$. The interpretation that the sodium leak represents an uncoupled pathway for sodium transport therefore suggests that the introduction of a cysteine in place of an alanine at 166 has altered the coupling characteristics of the transporter.

The importance of the molecular character of the residue at 166 to the function of the transporter is also supported by the estimates of turnover rate for the A166C mutant and the mutant following MTSEA exposure. Compared with wild type, the turnover rate was halved by the cysteine substitution ($\sim 23$ s$^{-1}$ versus $\sim 12$ s$^{-1}$) and the reaction with MTSEA caused an additional 3-fold decrease in the rate ($\sim 4$ s$^{-1}$). Due to the complexity of the kinetics for a system that couples the transport of two sodium ions to one glucose molecule, it is difficult to explain these changes in turnover rate in terms of the changes observed for the pre-steady-state data. Nonetheless, it is reasonable to speculate that the changes in the nature of the reorientation of the empty carrier along with sodium binding in response to membrane potential changes would be evidence that the same transition occurring during a transport cycle, in which membrane potential is held constant, has also changed. Since this transition is believed to be one of the rate-limiting steps in the transport cycle, such speculation is consistent with the turnover data.

Assuming that the effects of the MTSEA are due to reaction with cysteine 166, the results presented also provide information about the topology of the protein at position 166. Since MTSEA is a water-soluble compound that would not be expected to permeate across membranes, the residue at 166 must be placed either extracellularly or in some pore that is accessible from the extracellular compartment. This is important confirmation of the results of Turk et al. (21) which show that a 42-amino acid loop containing the native glycosylation site, when inserted between positions 169 and 170, is glycosylated by the Xenopus oocyte expression system. That particular mutant was reportedly non-functional while the A166C mutant we have shown is still able to mediate substantial $\alpha$MG-induced currents.

One important question we have been able to address is whether the effect of the MTSEA on A166C rSGLT1 is due to ateric effects or charge effects introduced by the ethyl amine group. Our data suggest that the positive charge on the amine is responsible for altering both the steady-state and pre-steady-state kinetics of the mutant transporter. Although we cannot determine directly whether MTSES is reacting with the transporter, the results showing that MTSES preincubation can protect against MTSEA incubation demonstrate that MTSES and MTSEA react with the same cysteine. Since MTSES had no effect on any of the functional characteristics of the transporter and given that ethyl sulfonate is negatively charged and bulkier than ethyl amine, we conclude that the MTSEA effect on transport kinetics and charge transfer are due to the addition of a positive charge in what must be a key location.

In conclusion, this study presents an application of cysteine mutagenesis coupled with reaction to MTs compounds to the elucidation of SGLT1 structure function relationships. We have provided evidence favoring the hypothesis that changes in the polarity of the molecular group at position 166 can bring about significant changes in both the pre-steady-state and steady-state kinetics of the transporter. A significant advantage to the approach with the MTSEA is that it allowed the comparison of functional parameters perturbed by a specific and rapid covalent reaction, with those same parameters measured in the same oocyte for presumably the same population of transporters before the perturbation took place. We speculate that application of this approach to other residues in SGLT1, particularly the region...
around position 166, can continue to provide valuable information about the mechanism of sodium-coupled transport. 

Acknowledgments—we thank R. Reithmeir, G. Gristein, and P. Back for helpful discussions.

REFERENCES
1. Wright, E. M., Loo, D. D., Turk, E., and Hirayama, B. A. (1996) Curr. Opin. Cell Biol. 8, 468–473
2. Hediger, M. A., Coady, M. J., Ikeda, T. S., and Wright, E. M. (1987) Nature 330, 379–381
3. Parent, L. Supplisson, S., Loo, D. D., and Wright, E. M. (1992) J. Membr. Biol. 123, 49–62
4. Chen, X. Z., Coady, M. J., Jackson, F., Berteloot, A., and Lapointe, J. Y. (1995) Biophys. J. 69, 2405–2414
5. Loo, D. D., Hazama, A., Supplisson, S., Turk, E., and Wright, E. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5767–5771
6. Panayotova-Heiermann, M., Loo, D. D., Lostao, M. P., and Wright, E. M. (1994) J. Biol. Chem. 269, 21016–21020
7. Lostao, M. P., Hirayama, B. A., Panayotova-Heiermann, M., Sampogna, S. L., Bok, D., and Wright, E. M. (1995) FEBS Lett. 377, 181–184
8. Panayotova-Heiermann, M., Loo, D. D., Kong, C. T., Lever, J. E., and Wright, E. M. (1996) J. Biol. Chem. 271, 10029–10034
9. Hirayama, B. A., Lostao, M. P., Panayotova-Heiermann, M., Loo, D. D., Turk, E., and Wright, E. M. (1996) Am. J. Physiol. 270, G319–G326
10. Martin, G. M., Turk, E., Lostao, M. P., Kerner, C., and Wright, E. M. (1996) Nat. Genet. 12, 216–220
11. Akabas, M. H., and Karlin, A. (1995) Biochemistry 34, 12496–12500
12. Javitch, J. A., Fu, D., and Chen, J. (1995) Biochemistry 34, 16433–16439
13. Stauffer, D. A., and Karlin, A. (1994) Biochemistry 33, 6840–6849
14. Javitch, J. A., Fu, D., and Chen, J. (1996) Mol. Pharmacol. 49, 692–698
15. Sarkar, G., and Sommer, S. S. (1990) BioTechniques 8, 404–407
16. Lund, A. H., Duch, M., and Pedersen, F. S. (1996) Nucleic Acids Res. 24, 800–801
17. Swick, A. G., Janicot, M., Chenval-Kastelic, T., McLenithan, J. C., and Lane, M. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1812–1816
18. Tate, S. S., Urade, R., Micanovic, R., Gerber, L., and Udenfriend, S. (1990) FASEB J. 4, 227–231
19. Hille, B. (1992) Ionic Channels of Excitable Membranes, 2nd Ed., Sinauer Assoc. Inc., Sunderland, MA
20. Hazama, A., Loo, D. D. F., and Wright, e. M. (1997) J. Membr. Biol. 155, 175–186
21. Turk, E., Kerner, C. J., Lostao, M. P., and Wright, E. M. (1996) J. Biol. Chem. 271, 1925–1934