Site-directed cysteine mutagenesis and chemical modification of the high affinity Na⁺/glucose transporter (SGLT1):

Elucidation of structure/function relationships underlying Na⁺ permeation through the transporter

by

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Abstract

Na⁺ cotransporters utilize the energy in the Na⁺ electrochemical gradient to drive the transport of sugars, amino acids, and a variety of other substrates into cells. This coupling of Na⁺ transport to the transport of another substance is a fundamental mechanism of energy transduction that has not been well defined at the molecular level. In this thesis, a combination of site-directed mutagenesis and voltage-clamp techniques is employed to identify structure-function relationships that underlie the binding of extracellular Na⁺ to the high affinity Na⁺/glucose cotransporter (SGLT1) expressed in Xenopus laevis oocytes. Results from the chemical modification of cysteines engineered into SGLT1 indicate that certain residues in the region between putative transmembrane helices IV and V form the entrance to a Na⁺-pore. Characterization of the steady-state and transient kinetics of another mutant in which a cysteine has replaced residue 156 in putative transmembrane helix IV suggests that this residue is located deep within this same Na⁺-pore. Together these results provide structural definition to the pathway that extracellular Na⁺ takes to its binding site in the SGLT1 transport protein.
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Dedication

To my parents
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Introduction
I-1. Introduction

I-1-1. Life, compartments, and transport

Life is very adept at creating order out of chaos. Sometimes it is so successful that it fools people into believing that it has managed to violate the law of increasing entropy, which states that the entropy of an isolated system must always increase. Of course, life does not in actual fact ever produce any contradictions to this important consequence of the second law of thermodynamics, instead it simply side-steps any confrontation by keying in on the concept of “isolation” as a condition to the law of increasing entropy. Life has figured out how to circumvent the “isolation” condition by creating systems of compartments and controlling the flow of “materials” between them. In other words, life is about compartments and arranging situations where order flows into some while chaos flows into others. Depending on the size of the “ruler” one uses to observe life, these compartments may be an ecosystem welcoming a migrating bird, a little boy eating an ice cream cone, an intestinal cell absorbing nutrients, or a synaptic vesicle filling up with neurotransmitters. In the most general case, the flow of “materials” that life controls, not only includes matter and energy, but also the flow of information since communication is simply a form of transport in which the influence of one compartment is passed to another.

It may be argued that all of the most fundamental compartments on which life is based are delineated by biological membranes composed of a lipid bilayer and membrane proteins. Chloroplasts, mitochondria, nuclei, other intracellular organelles and the cell itself are all packaged by biological membranes. Understanding the molecular mechanisms behind how substances and information are transported across these
biological membranes is one of the greatest challenges of modern molecular biology.

I-1-2. Biological membranes

The artificial lipid bilayers introduced by P. Mueller and D.O. Rudin (Meuller and Rudin, 1967) were permeable to water, gases such as oxygen and carbon dioxide, and small, relatively hydrophobic but water soluble molecules such as ethanol. They were not permeable to most water soluble molecules such as glucose, glucose phosphates, ATP, nucleosides, and amino acids, as well as larger molecules such as proteins. Ions and substances carrying a net charge were also impermeant and as a result the electrical conduction of these artificial membranes was exceedingly low.

Biological membranes in contrast to artificial lipid bilayers are much more dynamic structures owing to the presence of extrinsic (attached to the surface of the bilayer) and intrinsic (embedded within the bilayer) membrane proteins. Some of these proteins are key players in the transduction of information and signals across the membrane, while others participate in pinocytosis, endocytosis, and exocytosis, processes that allow proteins and large particles to sidestep the membrane barrier. Still others control the traffic of ions and small molecules across the lipid bilayer and are called channels, transporters or carriers. All of these membrane proteins, together with the lipid bilayer, are commonly conceptualized using the “fluid mosaic” model proposed by S. Singer and G. Nicholson (Singer and Nicolson, 1972).
I-1-3. Classification of transporters

The membrane proteins called transporters or carriers are traditionally classified according to the energetics of the transport they mediate in vivo. This classification distinguishes between three kinds of membrane transport processes - passive transport, primary active transport and secondary active transport.

1) Passive transport is a type of diffusion in which the ion or molecule moves down its electrochemical or concentration gradient during its passage across the membrane. Therefore, no metabolic energy is expended in passive transport. In simple diffusion the passive transport takes place without the help of a membrane transporter, whereas in facilitated diffusion the passive transport is mediated by an intrinsic membrane protein. Since many ions and molecules cannot by themselves overcome the barrier imposed by the hydrophobic core of the lipid bilayer, facilitated diffusion is an essential mechanism that maintains the semi-permeable quality of biological membranes. Examples of passive transport systems are the GLUT family of facilitative sugar carriers, anion exchangers (Band 3) and ion channels.

2) Primary active transport is the movement of ions or molecules against their electrochemical gradient by the direct expenditure of metabolic energy. Primary active transporters hydrolyse ATP and the energy released is coupled to transport. Examples of such proteins are V-type ATPases, F-type ATPases, and P-type ATPases.

3) Secondary active transport takes place when the movement of ions or molecules up their electrochemical gradient is coupled to the transport of an ion down its electrochemical gradient. An important role of certain primary active transport systems is to maintain a Na⁺ or H⁺ electrochemical gradient. The energy stored in this gradient is
then used by secondary active transport systems to move another ion or molecule across the membrane and accumulate it against a concentration gradient. Examples of such secondary active transport systems are the SGLT family of Na\(^+\) coupled solute transporters, the Na\(^+\)/H\(^+\) antiporter, and bacterial lactose permease.

It is important to note that passive transporters and secondary active transporters have enzyme like properties, but unlike enzymes, instead of participating in the making and breaking of covalent bonds, they catalyze surface chemical reactions that result in substrate translocation across the membrane. Simply put, their substrate is transported without being modified.
I-2. Na⁺/glucose transport

I-2-1. Brief history of Na⁺/glucose transport

In 1960, Crane presented for the first time some of his results on the Na⁺ requirement for glucose transport in intestinal brush border cells and hypothesized that the energy in the sodium gradient could be coupled to drive glucose transport (Crane, 1960). In the decade that followed, most experimental work was focused at confirming the Na⁺ dependence, and determining the substrate specificity and kinetic properties of the sugar transport system in various species and experimental model systems (Kimmich and Carter-Su, 1978; Goldner, 1973; Schultz and Curran, 1970; Crane, 1975). During this period, it became established that the Na⁺ chemical gradient was providing the thermodynamic driving force for sugar transport and that in addition to the intestine, the kidney tubule was another site of Na⁺-dependent sugar transport (Kleinzeller and Kotyk, 1961). Experiments studying the transport of other organic solutes extended the Na⁺ coupled transport concept to include a wide range of transport phenomena (Schultz and Curran, 1969).

Early sugar uptake experiments were performed using crude homogenates, tissue slices, whole organs, and micropuncture techniques (Silverman, 1991; Koepsell and Spangenberg, 1994). The development of subcellular fractionation protocols and methods to make brush border membrane vesicles represented a significant advance in the study of Na⁺-dependent sugar transport (Kinne et al. 1975). The brush border membrane vesicles allowed one to control solute concentrations on both sides of the membrane, and throughout the 70s and 80s, were used in equilibrium exchange experiments (Hopfer, 1975), initial uptake studies (Kessler and Semenza, 1983), and...
phlorizin binding studies (Moran et al. 1988) to examine the kinetic details of Na⁺-dependent sugar transport. The brush border membrane vesicles along with isolated epithelial cells (Carter-Su and Kimmich, 1980; Kimmich, 1981) were also used to establish that the driving force for transport was derived from not only the Na⁺ concentration gradient but also the membrane potential gradient. By the mid-70s there was consensus that Na⁺-dependent sugar transport was electrogenic and influenced strongly by changes in the membrane potential (Murer and Hopfer, 1974). However, by the mid-80s it also became clear that inherent limitations deriving from the inhomogeneous nature of the vesicles would prevent a final resolution of the various kinetic models for Na⁺-dependent sugar transport that had been proposed over the previous decade (Berteloot and Semenza, 1990).

Brush border membrane vesicles, in addition to being the experimental system in purely functional studies, were also used to examine structure/function relationships in semi-selective labeling (Neeb et al. 1985; Thomas et al. 1972; Peerce and Wright, 1984a; Peerce and Wright, 1984b), photoaffinity labeling (Ziegler et al. 1988), and radiation inactivation studies (Turner and Kempner, 1982; Beliveau et al. 1988; Stevens et al. 1990). In the 70s and 80s, the vesicles were also the starting point for a great many attempts at isolating the Na⁺/glucose cotransporter by solubilization-reconstitution (Koepsell and Seibicke, 1990; Koepsell, 1986). There was strong motivation for isolating the protein or proteins directly involved in Na⁺-dependent sugar transport and moving towards a detailed molecular description of coupled transport. Attempts were made to purify the Na⁺/glucose cotransporter from intestine, kidney and also the LLC-PK-1 cell line (Crane et al. 1977) using techniques such as selective solubilization in mild
detergents (Ducis and Koepsell, 1983; Koepsell et al. 1983), ion-exchange chromatography (Malathi and Preiser, 1983), phlorizin affinity columns (Silverman and Speight, 1986), ConA chromatography (Peerce and Clarke, 1990), and monoclonal antibodies (Koepsell et al. 1988; Wu and Lever, 1987). Due to a number of serious difficulties including the low abundance of the transporter in the starting materials, denaturation, aggregation and proteolysis during the detergent extraction process, none of these attempts ever succeeded in purifying the cotransporter sufficiently for amino acid sequencing.

In 1987, the rabbit intestinal Na⁺/glucose cotransporter (SGLT1) was cloned and sequenced using the novel approach of expression cloning with the Xenopus laevis oocyte (Hediger et al. 1987). This represented the first cloning of a mammalian Na⁺ cotransporter, and in the years to follow the application of Xenopus oocyte expression cloning has been used to identify the primary sequences of an amazing list of membrane transporters. Directly growing out of the rabbit SGLT1 sequence, were the sequences of a number of other Na⁺ cotransporters identified through the screening of cDNA libraries. Today, rabbit SGLT1 is one member of a family of homologous Na⁺/solute symporters (Reizer et al. 1994) that includes isoforms of SGLT1 from other species, low affinity Na⁺/glucose transporters (SGLT2), the Na⁺/myoinositol transporter, the Na⁺/nucleoside transporter, as well as the prokaryote Na⁺/proline and Na⁺/pantothenate transporters. It is important to note that the SGLT family of transporters share no sequence homology with the family of facilitated glucose transporters (GLUTs).

An important development in the functional characterization of the cloned Na⁺/glucose cotransporter was the application of electrophysiological techniques to study
the transporter expressed in *Xenopus* oocytes. Primarily this has been the two-electrode voltage clamp technique (Parent *et al.* 1992a), but the cut open oocyte technique has also been used to study SGLT1 (Chen *et al.* 1995). Electrophysiology has allowed for unprecedented control and manipulation of the membrane potential during the measurement of Na⁺-dependent sugar transport. In addition, the time scale over which data can be collected has allowed electrophysiology to identify for the first time transient currents associated with the Na⁺/glucose cotransporter. The pre-steady state kinetics derived from the measurements of these transient currents provide a means to calculate directly certain rate constants in the kinetic models (Wierzbicki *et al.* 1990). Moreover, the transient current measurements have led to estimates of the turnover rate (Zampighi *et al.* 1995; Loo *et al.* 1993), and hypotheses concerning the molecular basis underlying the membrane potential dependency (Panayotova-Heiermann *et al.* 1994; Wright *et al.* 1994b; Wright *et al.* 1994a). Over the last five years, electrophysiology with SGLT1 expressed in *Xenopus* oocytes has added remarkable insight to our understanding of Na⁺ coupled transport.

**I-2-2. Heterogeneity of Na⁺/glucose cotransporters**

The study of genetic defects in which renal or intestinal sugar transport is selectively impaired suggested the existence of heterogeneous systems of Na⁺/glucose cotransporters. In glucose-galactose malabsorption, intestinal sugar transport is knocked out, whereas kidney sugar transport is only slightly affected (Elsas *et al.* 1970; Lindquist *et al.* 1962; Meeuwisse and Dahlqvist, 1968). Conversely, in renal glycosuria, the renal transport is defective, while the intestinal transport of glucose is found to be normal (Elsas *et al.* 1970; Scriver *et al.* 1976). Work with brush border vesicles prepared from
intestinal scrapings and kidney tissue led to the hypothesis that there are at least two Na⁺/glucose cotransport systems in the kidney and that one of these is identical to the system found in the intestine (Turner and Moran, 1982a; Turner and Moran, 1982b; Silverman, 1991). When brush border vesicles were prepared from proximal tubules of different kidney regions, it was found that one of the renal transport systems, could be described as a low affinity high capacity system restricted mostly to the outer kidney cortex, and that the other, could be described as a high affinity low capacity system confined mainly to the outer kidney medulla. The high affinity system which shows a Na⁺:glucose stoichiometry of 2:1 is the same system found in the intestine while the low affinity system which exhibits a 1:1 stoichiometry is unique to the kidney (Turner and Moran, 1982c). With the cloning of the SGLT family of transporters, there is substantial evidence that SGLT1 defines the high affinity system and somewhat controversial evidence about the identity of the clone that defines the low affinity system. SGLT2 (SAAT1) (Mackenzie et al. 1994; Kong et al. 1993) and SGLT2 (Hu14) (Wells et al. 1992) are two clones that have been proposed to encode the 1:1 low affinity Na⁺/glucose cotransporter, as well, a distinct non-homologous clone, RS1 (Koepsell and Spangenberg, 1994; Veyhl et al. 1993) has been hypothesized to interact with SGLT1 and modulate its transport function.

1-2-3 Kinetic models describing SGLT1 transport

Prior to the cloning of SGLT1, a number of kinetic models had been proposed to explain the functioning of the high affinity Na⁺/glucose cotransport system. Among these models were Hopfer's proposal that Na⁺/glucose transport follows an ordered bi-bi mechanism (Hopfer, 1987; Hopfer and Groseclose, 1980), Crane's proposal that the
kinetic data supports a random bi-bi mechanism (Crane and Dorando, 1980; Crane, 1985), and the concept of Kessler and Semenza that the transporter is a gated channel with asymmetric kinetics (Kessler and Semenza, 1983; Semenza et al. 1984). None of these models explicitly included a membrane potential step in their calculations, although the model by Kessler and Semenza did propose that the membrane potential dependency of transport may arise from a "mobile gate" bearing at least one negative charge. At the time it was not possible to define quantitatively the relationship between membrane potential and transport activity and therefore qualitative relationships were emphasized.

All that changed however, with the expression of the cloned SGLT1 in Xenopus oocytes, the application of electrophysiology to that experimental system (Parent et al. 1992a; Birnir et al. 1991; Parent and Wright, 1993), and the development of whole cell patch clamp techniques to examine SGLT1 mediated currents in LLCPK-1 cells (Smith-Maxwell et al. 1990; Bennett and Kimmich, 1996; Bennett and Kimmich, 1992). These two approaches allowed for precise control over the membrane potential and thus a systematic and quantitative study of the membrane potential dependency of Na+/glucose transport. The results from such ongoing studies have challenged and continue to challenge theorists to explicitly incorporate membrane potential dependency into their kinetic models.

Currently there are two kinetic models which have been proposed for SGLT1 that explicitly incorporate into their rate constants membrane potential dependency, a 6-state model by Parent et al (Parent et al. 1992b), and an 8-state model by Kimmich et al (Bennett and Kimmich, 1996; Bennett and Kimmich, 1992; Kimmich and Randles, 1988). These two models are depicted in the following figure,
In the figure, states $[\cdot]'$ and $[\cdot]'''$ are the extracellular and intracellular facing conformations of the cotransporter, respectively; Na$^+$ represents sodium; and S represents sugar.

Both models take into account the established 2:1 Na$^+$:glucose stoichiometry and show an ordered binding mechanism with external Na$^+$ binding to the transporter prior to the binding of sugar. For simplicity, the 6-state model considers the two Na$^+$ binding events as a single transition. In contrast, the 8-state model takes into account previous work examining the Na$^+$ dependency of phlorizin binding (phlorizin is a non-transported competitive inhibitor) (Moran et al. 1988; Restrepo and Kimmich, 1986; Lever, 1984; Aronson, 1978) which suggests that the binding of sugar is preceded and followed by the binding of a Na$^+$ ion. The two models also differ in that the 6-state model incorporates a Na$^+$ transport pathway through the partially-loaded transporter, a so called "Na$^+$-leak" pathway. For simplicity, the 8-state model assumes that the "leak" pathways are small.
relative to the transport cycle involving the fully-loaded transporter and can therefore be ignored.

The 6-state and the 8-state models account for the membrane potential dependency of transport by making certain rate constants voltage dependent. The important assumption is that the voltage dependence of a given rate constant is directly related to the amount of charge which is translocated in the corresponding reaction step. According the microscopic description of the effect of voltage on ion driven cotransport systems formulated by Lauger and Jauch (Lauger and Jauch, 1986; Lauger, 1987), the charge translocation can result from three kinds of transitions, 1) movement of an ion along the transport pathway, 2) displacement of charged or polar ligand groups of the ion binding site, and 3) reorientation of charged or polar residues of the protein during a conformational transition. Mathematically, the influence of any charge translocation on a rate constant can be represented identically by an exponential predicted by Eyring rate theory (Keynes and Rojas, 1974), and the rate constant as a function of membrane potential takes the following form,

\[
k(V) = k_0 \exp(z\alpha FV/RT)
\]

where \(k_0\) is the rate constant in the absence of a membrane potential; \(z\) represents the equivalent charge crossing the whole membrane; \(\alpha\) (takes on values between 0 and 1) describes the asymmetry of the energy barriers; \(V\) is the membrane potential; \(F\), \(R\) and \(T\) are Faraday’s constant, universal gas constant and temperature, respectively.

Analysis of two-electrode voltage clamp data and patch clamp data in the context
of the 6-state and 8-state models have led to the assignment of charge translocations and thus membrane potential dependency to certain rate constants. Parent et al hypothesize that the membrane potential dependent transitions in the 6-state model are the reorientation of the empty transporter and the binding/debinding of Na⁺ (Parent and Wright, 1993). Bennett et al hypothesize that possible membrane potential dependent transitions in the 8-state model are both extracellular Na⁺ binding events and translocation of the free and fully loaded forms of the transporter (Bennett and Kimmich, 1996).

I-2-4. Transient currents and membrane potential dependent transitions

The existence of a membrane potential dependent transition predicts that transient currents associated with this transition may be observable during a voltage jump experiment. In this section I develop some basic theory for these transient currents and lay the groundwork for a discussion of the experimental transient currents that have been measured for SGLT1.

I consider a two-state system in which the transition from state 1 to state 2 is membrane potential dependent. Following the formalism derived from Eyring rate theory presented in the previous section, one can assign a charge translocation to the transition and write down the expressions for the forward and reverse rate constants,

\[ k_{12} = k_{012} \exp(z\alpha F V/RT) \quad \text{and} \quad k_{21} = k_{021} \exp(-z(1-\alpha) F V/RT) \]

where \( k_{012} \) and \( k_{021} \) are the rate constants in the absence of a membrane potential, and \( z \) is the equivalent charge (\( z > 0 \)).

In this two-state system, as \( V \to -\infty \) the probability that state 1 is occupied
approaches 1, and as $V \to +\infty$ the probability that state 2 is occupied approaches 1. If I consider a population of transporters that distribute themselves between states 1 and 2, then as $V$ goes from $-\infty$ to $+\infty$ there is a charge translocation that develops as transporters originally in state 1 end up going to state 2. This charge translocation is described by a Boltzmann equation of the following form,

$$Q(V) = \frac{Q_{\text{max}}}{1 + \exp((V - V_{0.5})zF/RT)} - Q_{\text{hyp}}$$

where $Q_{\text{max}}$ corresponds to the maximal charge transfer, $Q_{\text{hyp}}$ is $Q$ at the hyperpolarizing limit, $V_{0.5}$ is the voltage at which the charge movements are half completed, $z$ is the equivalent charge or apparent valence. The $V_{0.5}$ can be expressed in terms of the rate constants by,

$$V_{0.5} = \frac{RT}{zF} \ln \left(\frac{k_{012}}{k_{021}}\right)$$

A population of transporters in the two-state system will respond to a voltage jump by redistributing themselves between the two states according to the rate constants. Since every transition from state 1 to state 2 or vice versa is accompanied by a charge translocation, this redistribution is accompanied by a transient current. This transient (or pre-steady state) current is represented mathematically as follows,

$$I(t) = A \exp \left(-\frac{t}{\tau}\right)$$

where $A$ is a constant that depends on the steady-state distribution at the beginning of the voltage jump and $\tau$ is as follows,
\[ \tau = \frac{1}{(k_{12} + k_{21})} \quad \text{or} \quad \tau = \frac{1}{(k_{\text{012}} \exp(\alpha F V / RT) + k_{\text{021}} \exp(-\alpha F V / RT))} \]

If one assumes that the energy barriers are symmetrical (\( \alpha = 0.5 \)), then,

\[ \tau = \frac{1}{(k_{\text{012}} \exp(\alpha F V / 2RT) + k_{\text{021}} \exp(-\alpha F V / 2RT))} \]

Note that the time constant, \( \tau \) is only dependent on the final membrane potential and not the initial membrane potential. Finally, while the \( \tau \) vs \( V \) relationship is dependent on the asymmetry parameter (\( \alpha \)), the \( Q \) vs \( V \) relationship is the same irrespective of whatever value \( \alpha \) takes.

I-2-5. Transient currents exhibited by SGLT1

In 1992, when *Xenopus* oocytes expressing rabbit SGLT1 were subjected to electrophysiology, Parent et al observed transient currents in response to rapid changes in membrane potential (Parent *et al*. 1992a; Parent *et al*. 1992b). These transient currents (or pre-steady state currents) decayed much more slowly than the capacitive currents of the oocyte and were only observed in oocytes that were expressing SGLT1. Based on this and the observation that they were eliminated by saturating concentrations of either phlorizin or sugar, Parent et al tentatively concluded that these transient currents were due to the reorientation of the unloaded carrier within the membrane and/or the binding of Na\(^+\) to the transporter. The development of the 6-state kinetic model to explain both the steady state data and the transient currents, further led to the assignment of 70\% of the transient currents to the reorientation of the empty carrier and the remaining 30\% of the transient currents to the binding of external Na\(^+\). In addition, the internal Na\(^+\) binding
event was predicted to be voltage independent from the model simulations. It should also be noted that \textit{a priori} none of the other transitions in the 6-state model were assigned voltage dependency.

Using an improved two-electrode voltage clamp setup, others have observed similar transient currents for human SGLT1 and rat SGLT1 expressed in \textit{Xenopus} oocytes (Loo \textit{et al.} 1993; Panayotova-Heiermann \textit{et al.} 1995a). In these studies, the work of Parent \textit{et al} was extended by working out methods to isolate the transient currents specific to SGLT1 from the non-specific capacitive currents. Once this was done, it was found that the SGLT1-specific transient currents decayed according to a single exponential, and that integration of these transient currents yielded a charge transfer vs. voltage (Q vs. V) relationship that could be fit with the Boltzmann equation. A 3-state subset of the 6-state model by Parent was shown to account qualitatively for these observations.

\begin{center}
\[ \text{2Na} \rightarrow [C]' \rightarrow [C]'\rightarrow [C]'\rightarrow [C]'' \]
\end{center}

\textbf{3 State Model}

\begin{center}
Figure 2. Transitions responsible for the transient currents.
\end{center}

The 3-state kinetic model proposed to explain the transient currents predicts that the decay be described by two components or time constants. The fact that the decay of the transient currents could be adequately described by a single exponential was therefore duly noted. The proposed explanation was that the limited speed of the voltage clamp had probably obscured the second faster component which is associated with Na$^+$ binding.

Recently, the cut-open oocyte technique was used to reexamine the transient currents exhibited by human SGLT1 (Chen et al. 1996). In this study by Chen et al, the faster voltage clamp afforded by the cut-open oocyte resolved a second component in the decay of the transient currents. This fast component was characterized by a time constant that remained relatively invariant with respect to membrane potential. However, it was not the missing component predicted by the 3-state model because the ability to control the composition of the intracellular solution showed that both it and the slower component would persist even after sodium had been removed from both sides of the membrane. This second component could therefore not be associated with Na$^+$ binding and debinding. To account for this second component of the transient currents, the 3-state model was modified to include two transitions for the reorientation of the empty carrier.

The resulting 4-state model predicts that there are three components to the decay of the transient currents. The data from the cut-open oocyte experiments again fall one short of this prediction. Chen et al propose that the while the two slower components associated with empty transporter reorientation are observed by the cut-open oocyte technique, the third and much faster component associated with Na$^+$ binding and debinding remains too fast to be resolved.
Figure 3. Transitions responsible for the transient currents.

In their development of the 4-state model, Chen et al comment on the simplifying assumption made by Parent et al that the two Na\(^+\) binding events can be treated not only as equivalent but further as a single transition. In fitting the transient current data to the 4-state model, it was clear that despite the fact that two Na\(^+\) ions are transported in each transport cycle, it is uncertain whether both Na\(^+\) ions are involved in the generation of transient currents. Equally good fits to the 4-state model were obtained whether or not the assumption that two Na\(^+\) ions binding simultaneously was made. Therefore, the information contained in the time constants is not sufficient to distinguish between models with two Na\(^+\) ions binding simultaneously (Parent et al. 1992b) and other models where only one Na\(^+\) binds in the absence of sugar (Bennett and Kimmich, 1996).

I-2-6. SGLT2 (SAAT1) transient currents

Recently, the transient currents of SGLT2 (SAAT1) have also been examined and compared to those of SGLT1 (Mackenzie et al. 1996b). It was observed for SGLT2 that the Q vs. V relationship was shifted significantly toward positive potentials. At 100 mM external Na\(^+\), the Q vs. V relationship was shifted so much that it was not possible to obtain the part of the curve where Q saturates at positive potentials. However, the \(V_{0.5}\) was estimated to be +60 mV for [Na\(^+\)]\(_o\) = 100 mM from a plot of \(V_{0.5}\) vs. [Na\(^+\)]\(_o\) by
extrapolation. As with SGLT1, this relationship was linear, with $\Delta V_{0.5} = 96 \text{ mV}$ for every 10-fold change in external Na$^+$ (for rabbit SGLT1, $\Delta V_{0.5} = 98 \text{ mV}$ for $[\text{Na}^+]_o = 100 \text{ mM}$ to 10 mM (Hazama et al. 1997)). Though the data was not shown, the decay of the transient currents was fit with a single time constant (3-7 ms).

Clearly, more extensive characterization of the transients is needed, but the $V_{0.5}$ dependency on Na$^+$ being similar in both SGLT1 and SGLT2 deserves special attention. If the hypothesis that SGLT2 is a Na$^+$/glucose transporter with 1:1 Na$^+$:glucose stoichiometry holds true, then it naturally follows that the contribution to the SGLT2 transient currents can only be due to the binding of a single sodium ion. Furthermore, the SGLT1 transient currents, since they demonstrate the same $\Delta V_{0.5}$ for a given change $[\text{Na}^+]_o$ as those of SGLT2, must then be the result of the binding of one sodium ion and not two.
I-3. Transient currents exhibited by other membrane proteins

I-3-1. Voltage-gated ion channels

A number of membrane transport processes are strongly influenced by membrane potential changes. Perhaps the best understood is the voltage gating of ion channels where a depolarizing voltage pulse switches the channel from a closed to an open state (Bezanilla and Stefani, 1994; Catterall, 1995). This transition is believed to involve a positively charged transmembrane alpha helical domain known as S4, which in response to an appropriately large membrane depolarization is hypothesized to move outwards, generating a gating current and triggering the opening of the channel. Mutations of residues in S4 have been shown to alter gating current characteristics and change the voltage sensitivity of channel opening (Shao and Papazian, 1993). The movement in response to membrane depolarizations has also been directly measured using fluorescent probes attached to amino acid residues in S4 (Mannuzzu et al. 1996). Experiments indicate that the gating current arises from the translocation of the equivalent of six to twelve charges entirely across the membrane electric field and that most of these mobile charges are the charged residues within the S4 segment.

In contrast to the gating currents of voltage gated ion channels, the transient currents of SGLT1 have been shown to decay about 1-2 orders of magnitude more slowly and arise from the membrane translocation of the equivalent of 1-2 charges instead of a minimum of six. Furthermore, the theoretical analysis of the amino acid sequence of SGLT1 has not led to the identification of an S4 like segment.

I-3-2. Na⁺ cotransporters and H⁺ cotransporters

Other cotransporters such as the Na⁺/glutamate (Wadiche et al. 1995),
Na⁺/myoinositol (Hager et al. 1995), Na⁺/GABA (Mager et al. 1993), H⁺/myoinositol (Klamo et al. 1996), H⁺/sucrose (Boorer et al. 1996), H⁺/hexose (Boorer et al. 1994), and H⁺/oligopeptide (Mackenzie et al. 1996a) have also been shown to exhibit SGLT1-like transient currents in response to changes in membrane potential. Only the Na⁺/myoinositol cotransporter in this list of transporters exhibits sequence homology with SGLT1, and so the feature of transient currents may eventually turn out to be common to all Na⁺ and H⁺ coupled transporters. In this section I review in detail contributions that examine the transient currents exhibited by the Na⁺/glutamate, H⁺/myoinositol and H⁺/oligopeptide transporters in ways that may lead to a reevaluation of the current interpretation of the SGLT1 transient currents.

The transient currents mediated by the human glutamate transporter (EAAT2) expressed in Xenopus oocytes have been characterized by Wadiche et al using the two-electrode voltage clamp (Wadiche et al. 1995), and were found to be qualitatively similar to the transient currents exhibited by SGLT1. The EAAT2 transient currents were blocked by the transporter antagonist kainate with the same concentration dependence as the block of steady-state transport. This is analogous to what is observed with the SGLT1 inhibitor phlorizin and SGLT1 transient currents. Like the SGLT1 transients currents those mediated by EAAT2 also had Q vs V relationships that were well-fitted by the Boltzmann equation and these Q vs V relationships were found to shift towards negative potentials in response to a lowering of the external Na⁺ concentration. Finally, the EAAT2 transient currents were abolished by the removal of external Na⁺ which is the observation made for SGLT1 with intact oocytes using the two electrode voltage clamp (when the cut-open oocyte technique is used to eliminate internal Na⁺ this is not the
observation, see previous section).

Despite the similarity between the transients currents of EAAT2 and SGLT1, Wadiche et al do not resort to a version of the 3-state model proposed by Parent, in which both sodium binding and reorientation of the empty carrier contribute to the transient currents. A simpler model is suggested in which the transient currents arise solely from a voltage-dependent binding and debinding of external Na\(^+\) to a site which is within the membrane electric field. In the context of such a model, the observed shift in the Q vs. V relationship for a 50\% reduction in external Na\(^+\), and the membrane potential dependency of the time constant describing the transient currents were analyzed. Both analyses independently predicted that a single sodium ion traverses \(~40\%\) of the membrane electric field to get to its binding site on the EAAT2 transporter.

The transient currents mediated by the H\(^+/\)myo-Inositol cotransporter (cloned from *Leishmania donovani*) have also been characterized using the two-electrode voltage clamp by Klamo et al (Klamo et al. 1996). The essential observations, 1) that the transients were abolished by myo-inositol, 2) that the magnitude of the transients correlated with expression levels and 3) that the Q vs. V curve calculated from integration of the transient currents was well described by the Boltzmann equation, are all reminiscent of characteristics of the SGLT1 transients currents. To explore the validity of the two standard hypotheses concerning the origin of these transient currents, namely 1) that they are due to the voltage-dependent binding of protons or 2) that they are the result of empty carrier reorientations, Klamo et al experimented with the effect of temperature changes. It was assumed that global conformational transitions of proteins are likely to be more temperature-dependent than diffusional processes such as the
movement of an ion to its binding site. To summarize, the reduction of the temperature from 21 °C to 10 °C, while causing >85% decrease in the steady-state transport rate, was found to not produce as significant alterations in the kinetics of the transient currents. On this basis, it was proposed that the transient currents were not likely to reflect protein transitions associated with a rate-limiting step, instead they were more likely due to proton binding to sites in an occluded pore.

The intestinal H\textsuperscript{+}/oligopeptide cotransporter (PepT1) has been studied by Nussberger et al using the *Xenopus* oocyte expression system and the two-electrode voltage clamp (Steel *et al*. 1997; Nussberger *et al*. 1997). Using a variety of experimental protocols, not only was the effect of external pH on the transient currents studied, but the effect of internal pH was also examined. Consistent with the lowering of external Na\textsuperscript{+} concentration causing a shift in the SGLT1 Q vs. V curve to more negative potentials, the lowering of external H\textsuperscript{+} concentration caused a shift in the PepT1 Q vs. V curve to more negative potentials. The PepT1 Q vs. V curve was by inference (only the raw transient current data were shown) also shifted in this direction when the internal H\textsuperscript{+} concentration was raised. The finding that the transients were affected by intracellular protons led to an extension of the three state kinetic model (originally applied to PepT1 by Mackenzie *et al* (Mackenzie *et al*. 1996a)) to include a more detailed description of intracellular ion binding events. In this revised model, the binding of a proton at the extracellular or intracellular surface is followed by a conformational change that results in an outwardly or inwardly occluded state, respectively, that cannot reorient across the membrane without peptide substrate.
I-3-3. Na⁺/K⁺ ATPase

The reduction in the rate of net Na⁺/K⁺ pumping with hyperpolarization has been shown to be due to the membrane potential directly effecting the forward pump rate (Rakowski et al. 1997). Since this effect of membrane potential on pump rate is seen only in the presence of external Na⁺, it is believed that external Na⁺ deocclusion/reocclusion and/or the associated release/rebinding of Na⁺ ions are the likely voltage-dependent transitions undergone by a cycling Na⁺/K⁺ ATPase. Consistent with these ideas is the observation of transient currents mediated by the pump in response to voltage jumps (Rakowski, 1993). The Na⁺/K⁺ ATPase transient currents (with a rate of decay in the millisecond range) have been attributed to charge movements that occur under electroneutral Na⁺-Na⁺ exchange conditions (Rakowski, 1993). The dominant theme in recent work on the steady state and transient kinetics of the Na⁺/K⁺ ATPase has been the external access channel or “ion well” hypothesis, which places the external Na⁺ binding site within the membrane electric field (Gadsby et al. 1993). It has emerged that much of the charge translocation that occurs during a pump cycle is due to the movement of a Na⁺ ion to its external binding site rather than to the movement of charged residues accompanying some conformational change. In contrast to the view for SGLT1 proposed by the 6-state model, namely that the forward and backward rate constants for Na⁺ binding are both voltage dependent, the current view for the binding of external Na⁺ to the Na⁺/K⁺ pump is that only the forward (reocclusion) rate constant is potential dependent.

I-3-4. Na⁺/Ca²⁺ exchanger

There are significant differences between the electrophysiological properties of
SGLT1 and the Na⁺/Ca²⁺ exchanger studied by expression in oocytes and in cardiac cells (Hilgemann et al. 1997). For the Na⁺/Ca²⁺ exchanger, the rate of turnover is much higher (~5000/s) and the rate of decay for the transient currents is much more rapid (~10 μs). Nevertheless, the transients exhibited by the Na⁺/Ca²⁺ exchanger have been attributed to the binding and release of extracellular Na⁺ or a closely associated occlusion/deocclusion reaction. The basic concepts are very similar to those that have been proposed for the Na⁺/K⁺ ATPase.
I-4. SGLT1 structure/function

I-4-1. SGLT1 structure

With the cloning of rabbit SGLT1 the primary amino acid sequence of the transporter became available. Using standard algorithms that calculate hydrophobicity indices and hydrophobic moments, a folding model can be predicted from the information in this primary structure. Initially, the topology of SGLT1 was predicted to consist an intracellular N-terminus followed by 14 α-helical transmembrane domains connected by hydrophilic loops (Wright et al. 1992).

![Figure 4. Proposed topology of SGLT1.](image)

In 1996, the proposed topology was revised based on results from a series of glycosylation mutants of human SGLT1 (Turk et al. 1996). In this revised topology the N-terminus of SGLT1 is extracellular and there are 14 transmembrane α-helices that zig-zag across the lipid bilayer. Consistent with this proposed topology is the observation that wt SGLT1 is glycosylated at position 248 by oocytes and also during in vitro
translation by dog pancreatic microsomes (Hirayama and Wright, 1992). The cytoplasmic location of the hydrophilic region near the C-terminus has also been confirmed by immunogold microscopy with an antibody against residues 564-575 of rabbit SGLT1 showing that this antigenic site is on the cytoplasmic side of the microvillus membrane of kidney proximal tubule cells (Takata et al. 1991).

An interesting feature of the proposed topology is that a 14th transmembrane helix is formed by the final 20 residues in the SGLT1 sequence. These residues comprise a remarkably hydrophobic region that is absent in both the Na+/-proline and Na+/-pantothenate transporter sequences. Although it remains to be experimentally proven that this region exists as a transmembrane domain, the importance of it functionally has been suggested by the characterization of an SGLT1 construct tagged with the Stomatitus virus G protein epitope at the C-terminus (Turner et al. 1996). The epitope by immunohistochemistry was found to reside cytoplasmically, but since the epitope tagged SGLT1 demonstrated significant functional differences from wt, the structure of the transporter may have been disrupted.

I-4-2. Approaches to study SGLT1 structure/function relationships

Since the cloning of SGLT1, a number of approaches have been taken to elucidate structure/function relationships for the transporter. One approach has been to identify residues that may be critical to either the structure and/or function of SGLT1 by sequence analysis and/or alignment, and then to apply site-directed mutagenesis to test directly whether such residues are important. Another approach has been to identify the various mutations in SGLT1 that result in the clinical syndrome of glucose/galactose malabsorption. Since many of these mutations are point substitutions, this approach also
provides clues about what amino acids in the SGLT1 sequence may be important. Again the method of site-directed mutagenesis can be applied to directly test the hypotheses that arise from the identification of these naturally occurring mutations. A third approach has been to undertake the construction of chimeric transporters and SGLT1 truncation mutants in an effort to narrow down the regions essential for certain transporter functions. The section that follows reviews the developments in our understanding SGLT1 structure/function that have resulted from the application of these three approaches.

I-4-3. Site-directed mutagenesis studies

![Figure 5. Model of SGLT1 showing the mutations of site-directed mutagenesis studies.](image)

I-4-4. The D176A mutation alters transient current kinetics

The first site-directed mutagenesis study reported was one by Panayotova-Heiermann et al in which aspartic acid 176 in rabbit SGLT1 was mutated to either an asparagine or an alanine (Panayotova-Heiermann et al. 1994). The construction of these
mutants arose out the observation that the rabbit and human isoforms of SGLT1 demonstrate differences in their kinetics of charge transfer, and that the amino acid at position 176 is the only membrane-embedded residue (according to a theoretical SGLT1 topology) that is negatively charged in one but not the other isoform. In human SGLT1, 176 is an asparagine instead of an aspartic acid. The hypothesis was that certain membrane embedded charged residues were directly contributing to the charge movements exhibited by the transporter.

The above hypothesis was revised based on the results provided by the two mutants. The D176N mutant demonstrated the same kinetics of charge transfer as wt rabbit SGLT1, thus showing that the negative charge at 176 cannot account for the differences in the kinetics of charge transfer between the rabbit and human SGLT1 isoforms. The D176A mutant, however, exhibited Q vs. V and τ vs. V curves that were shifted 60 mV towards more negative potentials. This suggested to the authors that a polar group at position 176 may be playing a critical role in the kinetics of SGLT1 charge transfer and that perhaps polar residues and not necessarily charge residues are responsible for the membrane potential dependence of Na+/glucose transport.

In addition to changes in charge transfer, the D176A mutation produced a lower $K_i$ for phlorizin. The voltage dependency of the apparent sodium affinity was also changed while the apparent αMG affinity was unaffected. The 6 state kinetic model was able to simulate these changes, to a large degree, by adjusting the rate constants for the reorientation of the empty transporter.
The D427A mutation causes a protein trafficking defect

While the D176A mutant demonstrated altered transient kinetics, another alanine mutant (R427A) demonstrated altered trafficking to the plasma membrane. After changing arginine 427 to an alanine in rabbit SGLT1, Lostao et al subjected the resulting mutant transporter to the Xenopus oocyte expression system (Lostao et al. 1995). Although Western blot analysis of protein extracts from Xenopus oocytes showed that the R427A mutant was being made in quantities comparable to wt, no functional expression could be detected. Electrophysiology was unable to demonstrate either sugar induced currents or transient currents for the R427A mutant. This result was explained when freeze fracture analysis and immunocytochemistry localized the mutant protein to a membrane compartment just beneath the plasma membrane. The R427A mutation apparently blocked the delivery of the transporter to the oocyte surface. In a subsequent review by the same authors, it was noted that the proper trafficking of SGLT1 to the plasma membrane of oocytes is very sensitive to point mutations (based on the unpublished results of a large number of SGLT1 mutants) (Wright et al. 1996). This is a frustrating finding since any mutation that prevents the localization of the transporter to the plasma membrane also prevents the functional analysis of that mutant protein in intact cells.

In addition to D176 and R427 there are other charged residues that are predicted to be within a transmembrane helical domain. Three of them - E225, D273, and K321 were also mutated to alanine by Panayotova-Heiermann et al as reported in abstract form (Panayotova-Heiermann et al. 1995b). The E225A mutant apparently demonstrated the same transient and steady-state kinetics as wt. The D273A mutant showed reduced...
affinity for αMG and reduced transport activity by ~80%. Elimination of the lysine and hence positive charge at 321 dramatically decreased the apparent sodium and αMG affinities (Na⁺ Kₐ₅ from 10 mM to 70 mM, αMG Kₐ₅ from 0.2 mM to 40 mM) and increased the phlorizin Kᵢ from 10 μM to 0.7 mM.

I-4-6. Glucose/galactose malabsorption

First characterized almost 30 years ago, glucose/galactose malabsorption is characterized by the neonatal onset of severe, watery, life-threatening diarrhea that subsides almost immediately when glucose and galactose are removed from the diet (Wright et al. 1991). Mucosal biopsies from patients with glucose/galactose malabsorption show normal disaccharidase activities and normal amino acid transport. These same biopsies, however, are not able to accumulate either glucose or galactose. In a Swedish study examining the pedigree of six cases of the disease it was established that the genetic basis of glucose/galactose malabsorption is autosomal recessive (Melin and Meeuwisse, 1969).

The first mapping of glucose/galactose malabsorption to a mutation in the SGLT1 gene was reported by Turk et al (Turk et al. 1991). mRNA from intestinal biopsies taken from two sisters diagnosed with the disease was used to produce cDNA encoding SGLT1. Sequencing of this cDNA revealed a missense mutation that resulted in the substitution of aspartic acid 28 with asparagine. Oocyte expression studies confirmed that the mutation eliminated functional expression by preventing the trafficking of the transporter to the plasma membrane.
In a recent and much more extensive study, Martin et al (Martin et al. 1996) screened 33 patients with glucose/galactose malabsorption syndrome for mutations in the SGLT1 gene and identified 18 missense, 3 nonsense, 6 frameshift, and 4 splice-site mutations. Of the 18 missense mutations, 11 are of conserved residues in the SGLT1 family, and 13 involve the insertion or deletion of charged or polar residues. When expressed in *Xenopus* oocytes, 15 of the missense mutants demonstrated severely impaired sugar uptake activity, even though western blot analysis indicated that protein levels were comparable to wt. The other three missense mutants demonstrated only slightly reduced transport activity and are probably polymorphisms (two of these mutations occur in a patient whose disease is accounted for by a third mutation). Two of the missense mutants that demonstrated impaired transport activity, A304V and R499H, were subjected to additional analysis by electrophysiology. It was concluded that A304V
was not being packaged to the plasma membrane (no transient currents) and that R499H in addition to having defective trafficking (reduced $Q_{\text{max}}$), had a much lower sugar affinity than wt ($K_{0.5}$ 2.4 mM vs 0.3 mM).

In a recent abstract (Lostao et al. 1996), another missense mutation (Q457R) in SGLT1 was found to be the cause of glucose/galactose malabsorption in a Swedish family. When expressed in Xenopus oocytes the Q457R mutant mediated no transport activity detectable by $^{14}\text{C-}\alpha\text{MG}$ uptake or electrophysiology. Freeze fracture experiments, however, showed that it was packaged to the plasma membrane and the electrophysiology did detect transient currents. These transient currents demonstrated a similar Q vs. V relationship to those of wt, but their relaxation time constants were considerably longer. $\alpha\text{MG}$ and phlorizin inhibited the Na$^+$-leak current through the mutant protein with apparent affinities of 52 mM and 0.22 $\mu$M, respectively, which is about two orders of magnitude lower than those for wt. Perhaps the Q457R mutation eliminates the transport activity by reducing sugar affinity and preventing the conformational changes of the fully loaded transporter as it reorients within the membrane.

I-4-7. SGLT1 chimera and truncation mutant

Progress in identifying the residues involved in sugar binding has been advanced primarily by two papers, one reporting the characterization of a chimera of SGLT1 and SGLT2 (Panayotova-Heiermann et al. 1996) and another reporting the characterization of an SGLT1 truncation mutant (Panayotova-Heiermann et al. 1997). Together these two papers support the hypothesis that the C-terminal half of the transporter forms the sugar
permeation pathway. The chimera shows that sugar specificity is determined by the
second half of the protein, while the truncation mutant shows that the five terminal
transmembrane helices by themselves can form a specific low affinity glucose uniporter.

The chimera constructed by Panayotova-Heiermann et al consists of amino acids
1-380 of porcine SGLT2 (Mackenzie et al. 1994) and amino acids 381-662 of porcine
SGLT1(Ohta et al. 1990). Both clones were isolated from the LLCPK-1 cell line which
originates from pig kidney proximal tubule. The essential result reported for the chimera
is that the sugar specificity retained closely matches the one exhibited by SGLT1 but not
the one exhibited by SGLT2. Specifically, the chimera was capable of transporting
α-MG, D-glucose, D-galactose and 3-O-methyl-D-glucopyranoside. Recall that SGLT2
does not transport 3-O-methyl-D-glucopyranoside and transports D-galactose only very
poorly. The conclusion arrived at, therefore, was that recognition and transport of sugar
substrate is mediated by interactions distal to amino acid 380. Although other
experiments looking at the Na⁺ dependency of the sugar induced currents and the ability
of Li⁺ and protons to drive transport were reported, it was not possible to conclude as
definitively that the N-terminal half of the protein is responsible for cation binding and
transport. The best support for this view of cation binding came from the shape of the I-V
curve of the chimera which matched that of SGLT2 but not SGLT1.
Figure 7. A functional chimera of SGLT1 and SGLT2.

It is interesting to note that the chimera did not demonstrate a phlorizin inhibitable Na⁺ leak. Instead of inhibiting, phlorizin induced an inward current that was similar to the one induced by αMG. In fact, experiments with $^3$H-phlorizin showed that phlorizin was being transported by the chimera in a Na⁺ dependent manner.

Figure 8. A functional SGLT1 truncation mutant.

In the second paper by Panayotova-Heiermann et al, a truncation rabbit SGLT1 mutant consisting of amino acids 407-662 was expressed and characterized in *Xenopus laevis* oocytes (Panayotova-Heiermann et al. 1997). Western blot and freeze fracture
analysis showed that the truncation mutant was expressed and packaged to the oocyte plasma membrane, while electrophysiology showed that this expression did not produce any transient currents, Na\(^+\)-leak currents, or sugar induced Na\(^+\) currents. The truncation mutant, however, was not functionally inactive and the use of \(^{14}\)C-\(\alpha\)MG showed that it could transport sugar in a sodium independent manner. It was further shown that this sugar transport 1) has an apparent \(K_m\) of 50 mM for \(\alpha\)MG, 100 mM for D-glucose and > 100 mM for D-galactose, 2) has a turnover of \(~660/s\), and 3) is inhibited by phloretin (\(K_i\) \&~ \(0.5\) mM), but not by phlorizin (concentrations up to 1 mM). The conclusion was that helices 10-14 of SGLT1 together form the essential structure required to transport sugar and that the N-terminal region of SGLT1 (helices 1-9) may be required to couple this sugar transport to Na\(^+\) transport.

I-4-8. Structure function studies of the Na\(^+\)/proline transporter (PutP)

A number of prokaryote Na\(^+\) cotransporters share sequence homology with SGLT1 (Reizer et al. 1994), but only the Na\(^+\)/proline transporter has been subjected to structure/function and mutagenesis studies. I review here the progress that has been made with the Na\(^+\)/proline transporter, since developments in understanding the molecular mechanism of its transport activity may impact on our understanding of how SGLT1 functions. Since evolution has preserved certain aspects of their primary structure (Hediger et al. 1989), the expectation is that structure/function relationships pertaining to Na\(^+\) binding and the mechanism of transport coupling have also been preserved. To date, there have been no significant revelations regarding how the Na\(^+\)/proline transporter works at the molecular level, although some important clues have emerged.
The Na\(^+\)/proline transporter of E. coli (PutP) mediates the majority of proline transport into the bacterium with a Na\(^+\):proline stoichiometry of apparently 1:1 (Chen and Wilson, 1986). The sodium bound transporter demonstrates high affinity proline binding activity, and Li\(^+\) has been shown to be capable of substituting for Na\(^+\) (Yamato and Anraku, 1990; Chen et al. 1985; Mogi and Anraku, 1984b; Mogi and Anraku, 1984a). Since Na\(^+\) plus proline protects against NEM inhibition and Na\(^+\) alone partially protects, an active cysteine has been postulated to be involved in substrate and cation binding (Yamato and Anraku, 1988). Of the five endogenous cysteines, Anranku et al have mutated three of them, namely 281, 344, and 349, to serine, and found that while the C349S mutant remained sensitive to NEM, both C281S and C344S became resistant to inhibition by the sulphydryl reagent (Yamato and Anraku, 1988). This result together with observed changes in Na\(^+\) dependent proline binding led Anranku et al to conclude that the cysteine at 344 is functionally involved in substrate binding (Hanada et al. 1992). This is not to say, however, that cysteine 344 is the NEM reactive site, since the fact that the removal of either 281 or 344 fully eliminates NEM sensitivity actually argues that some other amino acid must be reacting. The likely candidates are the other two endogenous cysteines at positions 12 and 141, for which no data is as yet available. So what relevance does this have to SGLT1? Perhaps not too much relevance since there are no conserved cysteines between the PutP and SGLT1 sequences. On the other hand, with respect to the results presented in this thesis that show residues in putative transmembrane helix IV playing a role in Na\(^+\) binding, cysteine 141 in PutP is positioned in the transmembrane helix that aligns with this SGLT1 domain. Consequently, it is interesting to speculate that cysteine 141 is the NEM reactive site.
Besides cysteines 288 and 344, other amino acids in PutP have been shown to be important in Na⁺ binding. The R257C mutation results in a significantly higher Na⁺ apparent affinity and lower turnover rate (Ohsawa et al. 1988). The C141Y and G22E mutations both result in a lower Na⁺ apparent affinity (Yamato et al. 1990). It has been reported that the SGLT1 R300C mutation, which is equivalent to the PutP R257C mutation, is a loss of function mutation (Hediger and Rhoads, 1994). I note that the SGLT1 mutation equivalent to PutP C141Y would reside in putative transmembrane helix IV, and the one equivalent to PutP G22E has not yet been reported.

I-4-9. The cloning of a prokaryote Na⁺/glucose cotransporter (SgIS)

Recently a Na⁺/glucose cotransporter was cloned from *Vibrio parahaemolyticus*, a marine bacterium that requires Na⁺ for its growth (Sarker et al. 1996). This clone (designated SgIS) encodes a 530 amino acid protein that according to hydropathy analysis is a typical integral membrane protein with 10 to 14 transmembrane segments. Its amino acid sequence exhibits 31% identity and 75% similarity with human SGLT1, and 66% and 63% similarity with the Na⁺/proline and Na⁺/pantothenate transporters of *E. coli*.

Characterization of SgIS was initially carried out in a *Vibrio parahaemolyticus* strain that was deficient in the glucose-PTS system (Sarker et al. 1994). Subsequent to the cloning, characterization of SgIS was performed by transfecting an *E. coli* strain lacking glucose and galactose transport activity (Sarker et al. 1997). In both experimental systems, SgIS was shown to mediate Na⁺ dependent D-glucose and D-galactose transport. Surprisingly, the *E. coli* experiments demonstrated that phlorizin could inhibit the Na⁺/glucose transport, but not the Na⁺/galactose transport. It was also
found that Li⁺ could drive transport and that α-D-fucose, 2-deoxygalactose and αMG were substrates of the SgIS transporter.

I mention the SgIS transporter because it represents a potentially important development in the study of Na⁺/glucose transporter structure/function. The fact that SgIS shares significant functional and primary structural similarity to SGLT1 suggests that the structural features of SgIS important to Na⁺ binding, sugar binding, and substrate translocation may not be too different from those of SGLT1. Since purification of large quantities of SgIS may be more feasible because SgIS can be produced by bacteria, structural information about SgIS may be more forthcoming. In addition, there are functional assays that are possible with a transporter expressed in bacteria, that are difficult to perform on SGLT1 expressed in oocytes or mammalian tissue culture cells. The cloning of SgIS opens up many avenues for the study of Na⁺/glucose cotransport structure/function that are complementary to the current ones that have been developed for SGLT1.
I-5. Rationale

I-5-1. Why cysteine mutagenesis combined with sulphydryl group specific modification?

With the goal of elucidating structure/function relationships for SGLT1, I embarked on a research project that combined the techniques of site-directed mutagenesis with site-specific chemical modification. Each of these two experimental approaches have their own merits, but in combination these two approaches have proven to be extremely useful in identifying functionally important regions of proteins. In the case of SGLT1, research into structure/function is limited by the lack of high affinity antibodies. Mutating a critical residue in the SGLT1 sequence may eliminate functional expression, but without the ability to easily detect the mutant protein, it becomes impossible to distinguish this result from a mutation that simply leads to a trafficking defect. This is a very important consideration because it is not uncommon for a single site mutation to prevent a membrane transporter such as SGLT1 from reaching the plasma membrane (Wright et al. 1996). Moreover, as in the case of lac permease, residues which are absolutely critical for transport function may be very few and far between (Kaback, 1992). My use of mutagenesis was therefore not to necessarily find the amino acid positions critical for SGLT1 function, but rather it was to engineer special "target" amino acids into sites that were in close proximity to these critical sites. These "target" amino acids could then be reacted with various chemical reagents and the bulkiness or charge of the chemical groups thus introduced would perturb the transport function. A reasonable assumption was that each critical site would be surrounded by several positions into which "target" amino acids could be introduced, making these positions easier to find by
chance. Also, the two step approach of first introducing a “target” amino acid and then chemical modification of that “target”, nicely restricts any changes in function resulting from the chemical modification to those relevant to the folded transporter, and eliminates those that may pertain to transporter folding, processing, and trafficking. Lastly, the potential exists for studying the time course of any chemical reaction leading to functional changes and this may offer another dimension to the structure/function experiments.

I chose cysteine to be the “target” amino acid in my research based on preliminary experiments with wt rabbit SGLT1 expressed in Xenopus oocytes which showed that the sugar induced currents mediated by the wt transporter were not inhibited by a range of water soluble sulphydryl reagents. The wt rabbit SGLT1 sequence contains 15 endogenous cysteines and since there is no high resolution structural data showing which of them are buried and inaccessible, to be absolutely safe, it must be assumed that they all have the potential of reacting with aqueous sulphydryl specific reagents. However, the inability of various water soluble maleimides, mercurial reagents, and MTS derivatives to inhibit SGLT1 function suggested that if any endogenous cysteines were capable of reacting they were not critical to transport function and further were not located close to any amino acid that was. Therefore, instead of removing each of the endogenous cysteines to create a cysteineless mutant that perhaps would have its structure or function dramatically altered, I chose to introduce “target” cysteines into the SGLT1 sequence against the background of all the endogenous cysteines. I was relatively confident that any “target” cysteine informative about structure/function would not cause a large enough structural change to make a previously unreactive but functionally important or reactive
but functionally unimportant endogenous cysteine into one that is both reactive and functionally important. The prediction was that any such structural change, if at all possible, would likely completely inactivate the transporter and produce a null result. With these ideas in hand, I proceeded to introduce “target” cysteines by site-directed mutagenesis into SGLT1 and test the effect of various water soluble sulphydryl reagents on these mutants.

I-5-2. Why water soluble sulphydryl reagents?

Initially, I did not restrict myself to any particular sulphydryl reagent, although I did emphasize certain classes of reagents over others. Most importantly, I found that lipophilic reagents which could permeate across the lipid bilayer tended to cause non-specific and highly variable conductances to develop in the oocytes, consequently making the voltage clamp too unstable for good experiments. Therefore, the focus was on water soluble sulphydryl reagents, such as the methanethiosulphonate derivatives - MTSEA, MTSES and MTSET. The added benefit of using aqueous reagents was that I would be limiting myself to functionally important domains that were exposed to the extracellular space, for instance, the external binding sites of Na⁺ and sugar. The scope of possible interpretations of any experimental results was therefore simplified.
I-5-3. MTS compounds

Since methyl-methanethiosulphonate was introduced by Kenyon et al (Kenyon and Bruice, 1977), many methanethiosulphonate (MTS) derivatives have been developed (Akabas et al. 1992). Together they have found many applications in the biological sciences, most of them structure/function studies of a particular protein. For example, MTS derivatives have been used extensively to modify cysteines engineered into many of voltage-gated ion channels to map the pore region (Pascual et al. 1995; Kurz et al. 1995) react with cysteines in the acetylcholine binding site of the nicotinic receptor to determine the electronegativity of the region (Stauffer and Karlin, 1994), and inhibit a range of cysteine mutants made from a variety of ligand-gated ion channels (Javitch et al. 1995; Sun et al. 1996). With respect to cotransport proteins MTS derivatives have recently been applied to lac permease (Frillingos and Kaback, 1996) and the anion exchanger Band 3 (Casey, J., personal communication).

The chemical reaction that MTS derivatives undergo with the ionized sulphhydryl group of cysteines is outlined as follows,

\[ \text{CH}_3\text{SSR} + \text{R'S}^- \rightarrow \text{R'SSR} + \text{CH}_3\text{SO}_2^- + \text{H}^+ \]

where R varies with the particular MTS derivative and R'SH is the cysteine. Because it is a mixed disulphide reaction, MTS derivatives are absolutely specific for sulphhydryl
groups, unlike certain other commonly used sulphydryl group specific reagents such as the maleimides. The reaction tends to be pH sensitive, since the reaction proceeds only with the thiolate anion, and depending on the derivative, the chemical group linked by the disulphide bond may be small or bulky, charged or neutral, a spin label, a fluorescent probe.

Two of the most popular MTS derivatives are MTSEA (EA-ethylamine) and MTSES (ES-ethylsulphonate). Both are water soluble and relatively small molecules. The mixed disulphide they form is charged and roughly the same size as a bulky amino acid side chain. In the case of MTSEA, the group left behind on the protein is a positively charged ethyl amine and in the case of MTSES, the group left behind on the protein is a negatively charged ethyl sulphonate. Until recently both reagents were considered to be unable to permeate the oocyte plasma membrane. A report by Holmgren et al (Holmgren et al. 1996) suggests that MTSEA is able to cross that barrier, however, the large number of free sulphydryls (ex. Glutathione) available in the oocyte cytoplasm immediately “mops” up any MTSEA molecule that makes it into the oocyte.

I-6. Summary of the results of the project studying SGLT1 structure/function using cysteine scanning mutagenesis combined with MTS chemical modification.

I began introducing “target” cysteines into the region connecting putative transmembrane helices IV and V of rabbit SGLT1 using site-directed mutagenesis. Initially, I focused my attention on this region of SGLT1 because it exhibited a number of interesting features, 1) the region along with its adjacent transmembrane helices constituted one of the longest hydrophobic stretches in the SGLT1 protein sequence, 2) different algorithms that predict transmembrane helices from sequence information
produced different topologies for this region of the transporter, and 3) the 176 mutants characterized by Panayotova-Heiermann et al (Panayotova-Heiermann et al. 1994) suggested that this region may play an important role in the mechanism underlying the SGLT1 transient currents.

Almost immediately, I was fortunate enough to identify a cysteine mutant, in which the alanine at position 166 was replaced with a cysteine (A166C), that was rapidly and dramatically inhibited by MTSEA exposure. I tentatively concluded that I had found a “target” cysteine that would be informative about SGLT1 structure/function and proceeded to characterize the kinetics of the mutant. The results from that characterization are presented in chapter II, and show that not only were steady-state transport kinetics altered by the cysteine substitution, but also the kinetics of the transient currents. Moreover, the effect of the exposure to MTSEA on the transient (pre-steady state) currents suggested that with the chemical modification, I had found a means to perturb the transitions pertaining to Na⁺/binding and/or reorientation of the empty transporter.

Concurrent to the detailed characterization of the A166C mutant, I began a systematic cysteine scanning mutagenesis project of the region surrounding position 166. The results of that project are presented in chapter III, and show that “target” cysteines introduced at certain other positions in this region can be reacted with MTSEA and/or MTSES with the effect of inhibiting SGLT1 transport activity. Based on the distribution of these positions, and the nature of the MTSEA perturbations I speculate that amino acids 162-173 of SGLT1 form an α-helix with one face lining the pore through which
Na⁺ ions must diffuse on their way to the Na⁺ binding site on the transporter.

In chapter IV I present several experimental and theoretical treatments that were developed to further characterize the A166C mutant and help clarify, in terms of molecular mechanisms, the functional changes brought about by the cysteine substitution and MTSEA exposure. I apply the 6-state model developed by Parent et al (Parent et al. 1992b) to the kinetic data for the mutant and discuss how the model both succeeds and fails at explaining the changes brought about by the single cysteine substitution.

In chapter V I present results from two separate experiments that provide convincing evidence that residues in putative transmembrane helix IV and the region connecting it to the next transmembrane segment are lining the Na⁺ pore of SGLT1. In the first experiment some of the single cysteine mutations that result in MTSEA sensitivity are combined to produce double and triple cysteine mutants. The essential result presented shows that there is a progressive shifting of the Q vs V relationship to progressively more negative potentials as more and more charges are placed in this region of SGLT1 by reaction with MTSEA. I interpret this as a lowering of the affinity of Na⁺ binding to the transporter due the introduction of electrostatic interactions in the Na⁺ "access channel" or pore. In the second experiment, the characterization of the single cysteine mutant, T156C and its sensitivity to MTSEA demonstrate that position 156 is near the binding site of the second Na⁺. Results show that the chemical character of the side chain at position 156 can influence the binding of the second Na⁺ and conversely the binding of the second Na⁺ can affect the accessibility of the residue at 156 to MTSEA. I speculate here that the residue 156 is located deep in the Na⁺ pore and the Na⁺ binding site is comprised of residues in close proximity. Chapter VI concludes the thesis with a
discussion of the Na⁺-leak as it pertains to some of the cysteine mutants and the molecular mechanism underlying the transient currents.

To summarize, the work contained in this thesis represents the first documentation of a region of SGLT1 that is involved in Na⁺ binding. The results from combining the techniques of site-directed cysteine mutagenesis and sulphydryl group-specific chemical modification has provided results that support the hypothesis that residues from this region participate in defining the Na⁺ pore of SGLT1.
Replacement of Ala-166 with cysteine in the high affinity rabbit Na\(^+\)/glucose transporter alters transport kinetics and allows methanethiosulphonate ethylamine to inhibit transporter function
SUMMARY

An alanine to cysteine mutation at position 166 has been introduced by site-directed mutagenesis into the rabbit Na⁺/glucose transporter (rSGLT1). When expressed in *Xenopus laevis* oocytes, this mutant transporter (A166C rSGLT1) demonstrates a significantly lower apparent affinity for alpha-methyl glucoside (αMG) compared to the wild type transporter (apparent $K_m = 0.8$ mM vs. 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 cysteine allows the sulphydryl specific reagent, methanethiosulphonate 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 1mM 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.
INTRODUCTION

The Na⁺/glucose cotransporter is one member of a family of Na⁺-dependent transport proteins found in the brush border membrane of intestinal and kidney epithelia (Wright et al. 1996). It uses the Na⁺ electrochemical gradient to drive the transport of sugar into epithelial cells and is specifically inhibited by phlorizin. Since being cloned in 1987 from a rabbit small intestinal cDNA library (Hediger et al. 1987), 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 Na⁺ currents mediated by the transporter, and from such measurements substrate affinities as well as the Na⁺:glucose coupling stoichiometry have been determined (Parent et al. 1992a; Chen et al. 1995). 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 Na⁺ association/dissociation and the reorientation of the empty transporter from an outside facing to inside facing conformation (Loo et al. 1993).

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 (Panayotova-Heiermann et al. 1996; Lostao et al. 1995). 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 (Panayotova-Heiermann et al. 1996).
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 (Hirayama et al. 1996). 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 Na\(^+\) 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 galactose malabsorption patient it was found mutated to a
threonine (Martin et al. 1996). Ala-166 is conserved across the rabbit/rat/human isoforms
of SGLT1 as well as SGLT2 and the Na\(^+\) myoinositol transporter. This is not surprising
given the high degree of sequence identity for these members of the Na\(^+\) 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 methanethiosulphonate ethylamine
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 (Akabas and Karlin, 1995; Javitch et al. 1995; Stauffer and Karlin, 1994; Javitch et al. 1996; Sarkar and Sommer, 1990; Lund et al. 1996; Swick et al. 1992; Tate et al. 1990; Hille, 1992). 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.

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 was subcloned into the remaining EcoRI site. The A166C mutation was introduced into this construct using the megaprimer method of PCR mutagenesis (Sarkar and Sommer, 1990) with 5'-TCGGAGCCTCTCTGTGGT-3' as the sense primer, 5'-TACCCAGACAGAATCGAGCCGCTT-3' as the antisense primer, and 5'-GGATGAAGATGCATCCGGAAAAGATG-3' as the mutagenic primer. These primers were used to make a mutation containing PCR product that was digested with BclI and then ligated to BclI digested PMT3-rSGLT1 using a cycle ligation protocol (Lund et al. 1996). The mutation and the stretch of DNA between the two BclI sites was verified by dideoxy chain termination DNA sequencing using the Pharmacia 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 - Xenopus laevis_ frogs were anaesthetized 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 Chemical Co. St. Louis, MO) prepared in modified Barth’s saline (MBS) for 60 to 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, 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, 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 (Swick et al. 1992). The injected oocytes were kept in MBS supplemented with 2.5 mM Na pyruvate for 2-3 days before being transferred to 96 well plates to be incubated individually another 16-24 hr. The incubation solution from each oocyte was then tested for secreted alkaline phosphatase activity following the protocol of Tate et al (Tate et al. 1990). 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 technique (Hille, 1992). 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 uptake buffer was 100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 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 kHz, 5 point Gaussian filter prior to curve fitting or the calculation of steady state parameters. Curve fitting was done either using the Simplex 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 ms and 0.7 ms.

RESULTS

Fig. 1 shows the current responses to a series of 100 ms voltage steps (-150 mV 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 (Wright et al. 1996), the presence of αMG in the bath solution induces an inward Na⁺ current which is not observed in water injected oocytes. Moreover, A166C rSGLT1 expression results in current responses which
consist of a capacitive 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 Na⁺ binding and the reorientation of the empty carrier (Loo et al. 1993). As with wild type SGLT1, this pre-steady state current is abolished by the addition of αMG. Panel C 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 Na⁺-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 afterwards in the presence of αMG; the Na⁺-leak is defined experimentally as the current inhibited by saturating phlorizin concentrations in the absence of αMG. In panel D we compare the Na⁺-leak of the A166C mutant and wild type SGLT1 by normalizing with respect to I_{max} (see fig. 2A). Clearly, the A166C mutant exhibits a much smaller Na⁺-leak at negative membrane potentials.

Since the αMG induced currents for A166C rSGLT1 were generally speaking comparable in magnitude to 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 re-plotted as functions of αMG 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 αMG has been lowered by the alanine to cysteine substitution. In
Figure 1 Sugar induced inward Na$^+$ currents mediated by A166C rSGLT1. Shown here are representative current responses obtained from an oocyte injected with the pMT3 A166C rSGLT1 that was positive according to the SEAP assay. The on and off current responses to a series of thirteen 100 ms voltage pulses (-150 mV to 90 mV in 20 mV increments) are shown as overlapping traces. The holding potential was -50 mV. In panel A the recordings were made in a 100 mM Na$^+$Cl buffer and in panel B the recordings were made in the same buffer containing 10 mM αMG. After calculating the differences in the current traces in panels A and B, the mean values of these current differences corresponding to the final few ms of the voltage pulse (i.e. the steady state currents) are plotted as a function of voltage (panel C). Also plotted in panel C is the difference in the current measured in the absence and in the presence of 0.2 mM phlorizin for this same oocyte. Referred to as the Na$^+$-leak current, this steady state phlorizin inhibitable current in panel D is compared to the Na$^+$-leak current obtained from an oocyte expressing wt SGLT1. The currents have been normalized by dividing by $I_{max}(-150 \text{ mV})$, the current obtained at saturating αMG for $V_i = -150 \text{ mV}$. 
experiments where the \(\alpha MG\) induced currents were determined for a series of \(Na^+\) concentrations ranging from 0 mM to 100 mM, data was obtained which showed that the mutation did not appear to affect stoichiometry or the apparent \(Na^+\) affinity. In fig.2C a representative experiment is shown in which the \(Na^+\) dependency of a 1 mM \(\alpha MG\) induced current at -30 mV, -50 mV 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 steady state \(\alpha MG\) induced currents 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 \(\alpha MG\) (10 mM) as at lower concentrations. Although in the absence of \(\alpha MG\) we could not directly monitor the time course of inhibition, MTSEA exposure under such conditions resulted in the 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 CholineCl (data not shown).

The inhibition by the MTSEA did not eliminate the \(\alpha MG\) induced currents although the kinetics of the curve in fig. 3 indicate that the reaction has gone to completion. Analysis of the \(\alpha MG\) currents remaining after exposure to MTSEA (fig. 4) demonstrates that the effect of MTSEA is both on the \(\alpha MG\) apparent affinity as well as the \(I_{max}\) of the transporter.

In order to further examine the effect of the cysteine substitution and the MTSEA inhibition, we undertook a series of experiments to examine the pre-steady state currents
Figure 2A Sugar induced Na⁺ 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 Na⁺ and a varying amount of aMG (in mM, 0.1, 0.5, 1.0, 2.0, 5.0, 10.0). Current responses were recorded before and after the addition of each concentration of aMG for a series of 100 ms voltage steps. The steady state current (i.e. the currents recorded during the final few ms of the voltage pulse) induced by the aMG was taken as the difference between these two measurements. These aMG induced currents as a function of aMG concentration are shown here fitted to the Michealis Menton relationship $I = I_{\text{max}} \frac{[\text{aMG}]}{K_{m} + [\text{aMG}]}$, where $I_{\text{max}}$ is the current at saturating $[\text{aMG}]$ and $K_{m}$ is the apparent aMG affinity.
Figure 2B Voltage dependence of the αMG affinity for A166C and wt rSGLT1. The data presented here is mean of three experiments for A166C and three experiments for wt rSGLT1. The values for the αMG $K_m$ were derived from fitting data to the Michealis Menton relationship as described for fig. 2A. The error bars represent standard deviations.
Figure 2C  Na⁺ activation of the steady-state sugar induced currents. This representative experiment shows the Na⁺ dependence of the currents induced by 1 mM αMG. Recordings were made in bath solutions in which varying amounts of Na⁺ was replaced with equimolar amounts of Choline. The currents depicted are the differences in the current recordings made at a particular Na⁺ concentration before and after the addition of 1 mM αMG. The data is fitted to the Hill equation, $I = I_{\text{max}} \frac{[\text{Na}^+]^n}{([\text{Na}^+]^n + K_{0.5}^n)}$, where $I$ is current, $I_{\text{max}}$ is the maximal current at saturating [Na⁺], $K_{0.5}$ is the [Na⁺] at which $I = I_{\text{max}}/2$ (i.e. apparent Na⁺ affinity), and $n$ is the Hill coefficient. For -70 mV, the curve fitting gives $I_{\text{max}} = -134 +/- 6$ nA, $K_{0.5} = 19.9 +/- 2.0$ mM, $n = 1.32 +/- 0.1$; for -50 mV, $I_{\text{max}} = -115 +/- 6$ nA, $K_{0.5} = 24.2 +/- 2.2$ mM, $n = 1.66 +/- 0.15$; for -30 mV, $I_{\text{max}} = -87 +/- 4$ nA, $K_{0.5} = 28.2 +/- 2.1$ mM, $n = 2.5 +/- 0.3$; errors are the errors of the fit.
Figure 3 Time course of the MTSEA inhibition of αMG induced currents. A representative experiment is shown for an oocyte expressing A166C rSGLT1. The oocyte was voltage clamped and the bath solution contained 1 mM αMG. At the time indicated 1 mM MTSEA was added to the bath solution and within seconds the αMG induced currents began to decrease. The inhibition appears to be complete within approximately one minute after the addition of the MTSEA. Upon removal of the MTSEA from the bath solution there was no change in the remaining αMG induced currents and upon removal of the αMG from the bath solution the current recordings went back to the baseline measured at the beginning of the experiment in the absence of αMG.
of the mutant transporter before and after MTSEA exposure. Fig. 5 (panels A and B) show the transient currents observed for an oocyte expressing A166C rSGLT1 prior to and subsequent to reaction with MTSEA. The traces are the difference in the currents obtained in the presence and absence of 200 μM phlorizin. Phlorizin is a specific inhibitor of the transporter that has been shown to effectively eliminate the transients associated with the transporter and provides a means to measure and subtract the non-specific capacitive currents (Parent et al. 1992a; Loo et al. 1993). For purposes of clarity and comparison, we overlay the transient currents for the +90 mV voltage step from panels A and B in the C panel of fig. 5.

MTSEA affected the rate of decay of the transient currents exhibited by the A166C mutant. Qualitatively, the decay of the on currents is faster after MTSEA exposure. When the on currents are fitted to a first order exponential decay equation, time constants can be obtained which demonstrate the effect quantitatively (fig. 5, panel D).

The transient currents for each voltage pulse (V) can be integrated to give a value for the quantity of charge transferred (Q) as a result of the change in membrane potential. Q as a function of V has been previously demonstrated to be adequately described by the Boltzmann equation (Loo et al. 1993). Figure 6, panel A shows how the Q vs V relationship for A166C is changed by exposure to MTSEA and panel B compares the normalized Q vs V relationship for wt, A166C before and after MTSEA inhibition.

The Qmax obtained from fitting data to the Boltzmann relation correlates with the number of transporters expressed in a given oocyte. It has been shown that Imax/Qmax can provide an estimate of the maximal rate of transporter turnover (assuming a
Figure 4A The effect of MTSEA on the αMG affinity of A166C. The mean of αMG
$K_m$ values determined from fitting data from three separate experiments (i.e. three oocytes
expressing A166C that have been exposed to 1 mM MTSEA) is plotted as a function of
membrane potential. For comparison we have re-plotted the data for A166C (before
MTSEA exposure) from fig. 2B. The error bars are standard deviations.
Figure 4B  The effect of MTSEA on the $I_{\text{max}}$ for the $\alpha$MG induced current mediated by A166C. Measurements of the $\alpha$MG induced currents at varying concentrations of $\alpha$MG were made in an oocyte expressing A166C. The oocyte was then exposed to 1 mM MTSEA and the same measurements of $\alpha$MG induced currents at varying concentrations of $\alpha$MG were made. From fitting this data to the Michaelis Menton relationship, values for $I_{\text{max}}$ were determined and plotted as a function of membrane potential. The error bars represent the errors of the fit.
Figure 5 The transient currents expressed by A166C and the effect of MTSEA exposure. (panel A) In an oocyte expressing A166C, current responses to 100 ms voltage pulses were recorded before and immediately after the addition of 0.2 mM phlorizin. The difference in these current responses are plotted as a series of 13 overlapping traces corresponding to the 13 voltage pulses ($V_0$-150 mV to +90 mV in 20 mV increments, $V_h$ was -50 mV). (panel B) The oocyte was then exposed to 1 mM MTSEA for 5 min and the protocol with the 0.2 mM phlorizin was repeated. Again the difference in the current responses before and after the addition of 0.2 mM phlorizin are plotted as overlapping traces. (panel C) The traces corresponding to $V_t$ = 90 mV from the upper and middle panels are re-plotted together. (panel D) The on currents corresponding to $V_t$ = 50, 70, 90 mV were fitted to a single exponential decay function and time constants were obtained. The graph compares the time constants for wt, A166C and A166C after MTSEA exposure; error bars are the standard deviations (n = 6).
stoichiometry of 2:1 and that 2 charges per transporter are translocated by the transient currents) (Loo et al. 1993). Using the $I_{\text{max}}$ for -150 mV, we have found that wt SGLT1 has a maximal turnover rate of 22.8 +/- 0.5 s$^{-1}$ (n=3), while the A166C mutant exhibits a turnover of 12.3 +/- 0.3 s$^{-1}$ (n=5). Since the $I_{\text{max}}$ of A166C before and after MTSEA inhibition can be determined in the same oocyte, the change in turnover rate caused by the MTSEA can be determined directly. Assuming that the number of transporters has not changed during the MTSEA exposure, the decrease in turnover is directly proportional to the decrease in $I_{\text{max}}$. Hence, MTSEA results in a 3-fold decrease in turnover rate ($I_{\text{max}}$ before MTSEA/$I_{\text{max}}$ after MTSEA is ~3).

To explore further the nature of the MTSEA effect on the mutant, we tested a related compound, methanethiosulphonate ethylsulphonate (MTSES). Like MTSEA, MTSES reacts specifically and rapidly with thiols to form mixed disulphides. However, the unreacted MTSES in solution possesses a negative charge instead of a positive charge and the mixed disulphide it forms is the negatively charged thiol ethylsulphonate instead of the positively charged thiol ethylamine. We first confirmed that MTSES at 1 mM and 10 mM had no effect on wtSGLT1 steady state or pre-steady state kinetics (data not shown). Then interestingly we found that MTSES at these concentrations likewise had no effect on the kinetics of the mutant (data not shown). Incubation with 10 mM MTSES did however protect against a subsequent inhibition with MTSEA. Fig. 7 shows that for progressively longer pre-treatments with MTSES followed by washout of the MTSES there is a decreasing degree to which MTSEA exposure can inhibit the αMG induced steady state currents. These findings indicate that MTSES can also react with the

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cysteine at position 166, but that the mixed disulphide it forms does not perturb transporter function.

![Graph showing charge transfer Q as a function of test potential V.]

Figure 6A Charge transfer Q as a function of the test potential V, for A166C before and after MTSEA inhibition. Data is shown for a representative oocyte expressing 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 Vr. The holding potential Vh was -50 mV and prior to integration the current transients were baseline corrected using the mean current values obtained over the final few ms of the voltage pulse. The data is fitted to the Boltzmann relation, 

$$Q = Q_{\text{max}}[1 + \exp((V - V_{0.5})zF/RT)] - Q_{\text{hyp}}$$

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}} = 37347 +/- 468 pC, V_{0.5} = 17.44 +/- 0.91 mV, z = 1.01 +/- 0.03, Q_{\text{hyp}} = -2759 +/- 158 pC. For A166C after MTSEA exposure, Q_{\text{max}} = 35054 +/- 355 pC, V_{0.5} = -1.14 +/- 0.79 mV, z = 1.03 +/- .03, Q_{\text{hyp}} = -4450 +/- 174 pC.
Figure 6B Normalized charge transfer $Q_{\text{norm}} = (Q - Q_{\text{hyp}})/Q_{\text{max}}$ as a function of $V_t$.

Data is shown for the mean of 3 experiments for wtSGLT1, 8 experiments for A166C and 3 experiments for A166C after MTSEA inhibition. Error bars represent the standard deviations; superimposed on the data are the corresponding Boltzmann relation curves.

For wt, $V_{0.5} = -2.54 +/- 0.70$ mV, $z = 1.07 +/- 0.03$; for A166C, $V_{0.5} = 19.53 +/- 0.95$ mV, $z = 1.00 +/- 0.03$; for A166C after MTSEA, $V_{0.5} = 1.59 +/- 0.78$ mV, $z = 0.99 +/- 0.03$. 
Figure 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 αMG 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 αMG. Each of the four oocytes exhibited approximately the same current levels induced by 1 mM αMG 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 extracellular 16 amino acid loop connecting transmembrane helices IV and V of SGLT1 (Turk et al. 1996). To date, the only experimental data ascribing function to any part of this loop comes from a study by Panayotova-Heiermann et al. in which the aspartic acid at position 176 (located at the TM V end of the loop) was mutated to an alanine (Panayotova-Heiermann et al. 1994). In this study, the D176A mutation was shown to result in 1) an increased rate of decay for the on current transients corresponding to membrane depolarizations and 2) a shift of the $Q$ vs $V_i$ 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 $Q$ vs $V_i$ 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 on transient currents ($\tau_{on}$) change and the $Q$ vs $V_i$ curve shifts.

Referring to the modelling of SGLT1 current transients by Loo et al. (Loo et al. 1993), 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 Na⁺ through the membrane electric field. For membrane depolarizations, the
transition would be the dissociation of Na⁺ followed by reorientation of the unloaded
transporter from outside to inside facing (i.e. Na⁺C_out => C_out => 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 τ_on (for V_t = +90 mV) than wild
type implies that the cysteine substitution has caused a decrease in the rate of this Na⁺C_out
=> 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 τ_on (for V_t = +90 mV) and shifts the Q vs V_t
curve along the voltage axis towards more depolarizing potentials such that the
normalized Q vs 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⁺C_out => 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 αMG apparent affinity, an observation which indicates that the mutation has affected either the true αMG 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 Na⁺ activation of sugar binding, perhaps Na⁺ 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 αMG and phlorizin, the cysteine at 166 reacting with the MTSEA is unlikely to be located within either the αMG or the phlorizin binding sites. The decrease in the αMG 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 Na⁺ binding to activate αMG binding and then transport.

Another piece of evidence that supports the hypothesis that 166 is located in a region important to Na⁺ binding and the coupling of this event to sugar transport is the order of magnitude lower Na⁺-leak exhibited by the A166C mutant as compared to that for wild type. The comparison holds for the case where the leak was normalized to \( I_{\text{max}} \) and also for the case where the leak was normalized to \( Q_{\text{max}} \). The interpretation that the Na⁺-leak represents an uncoupled pathway for Na⁺ 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 to wild type, the turnover rate was halved by the cysteine substitution (~23 s\(^{-1}\) vs ~12 s\(^{-1}\)) and the reaction with MTSEA caused an additional three fold decrease in the rate (~4 s\(^{-1}\)). Due to the complexity of the kinetics for a transport system that couples the transport of two Na\(^+\) 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 Na\(^+\) 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 accessible from the extracellular compartment. This is important confirmation of the results of Turk et al 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 (Turk et al. 1996). That particular mutant was reportedly non-functional while the A166C mutant we have shown is still able to mediate
substantial αMG induced currents.

One important question we have been able to address is whether the effect of the MTSEA on A166C rSGLT1 is due to steric 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 pre-incubation 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 sulphonate 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 favouring 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 Na⁺ coupled transport.
Cysteine scanning mutagenesis of the region between putative transmembrane helices IV and V
Summary

By combining the techniques of site-directed mutagenesis and chemical modification of specific amino acid side chains, I have elucidated structure/function relationships for Na⁺/glucose transport. Beginning with the cloned rabbit transporter (rSGLT1), each amino acid in the region (162-173) between putative transmembrane helices IV and V was replaced individually with cysteine (Cys). The mutant proteins were expressed in *Xenopus Laevis* oocytes and using the two electrode voltage clamp method, the effect of the Cys substitution on the function of the transporter was assayed. All of the Cys mutants demonstrated some degree of sugar induced sodium currents and were therefore processed to the plasma membrane of the oocytes. Some of the Cys mutants demonstrated alterations in both their steady-state and pre-steady state kinetics that suggested a role for this region in determining the Na⁺ binding and voltage sensing properties of the transporter. Furthermore, a subset of the Cys mutants became sensitive to inhibition by certain water soluble methanethiosulphonate derivatives, compounds which specifically target and modify the sulphydryl group of cysteines. Since the wild type rSGLT1 was previously determined to be insensitive towards such compounds, it was concluded that the introduced cysteine was being reacted and the resulting modification was the reason for the inhibition. Analysis of the nature of the inhibition provides evidence that this region of the protein may be involved in Na⁺ binding. Finally, the distribution of the cysteines in the Cys mutants that were sensitive to the MTS derivatives suggests that this region forms an α-helix.
Introduction

The Na⁺/glucose cotransporter (SGLT1) is a multiospanning membrane protein that uses the Na⁺ electrochemical gradient to drive the transport of sugar across intestinal and renal brush border membranes. It was the first in a series of highly homologous Na⁺ coupled cotransporters to the cloned (Hediger et al. 1987), and from a functional point of view, is the most extensively characterized member of this protein family. SGLT1 isoforms from several species have been expressed in Xenopus oocytes and their transport kinetics subjected to detailed analysis by electrophysiology (Hazama et al. 1997; Panayotova-Heiermann et al. 1995a; Loo et al. 1993; Hirayama et al. 1996). Using the oocyte expression system, the transporter’s 2:1 sodium:glucose coupling ratio has been confirmed by direct measurement of the respective fluxes and also by studying the reversal potential of the sodium currents as a function of sugar concentration (Lee et al. 1994; Chen et al. 1995). Charge movements pertaining to Na⁺ binding and the reorientation of the empty transporter within the membrane have also been studied and a 6-state kinetic model of the transporter has been proposed (Parent et al. 1992b). In addition, an 8-state kinetic model has been used to explain patch clamp data on the endogenous Na⁺/glucose cotransporter of a kidney cell line which by all criteria is an SGLT1 isoform (Bennett and Kimmich, 1996). The substantive difference between the two kinetic models is that the 8-state model considers the two sodium binding events to be non-equivalent, the second sodium binding event occurring after the binding of glucose.

Compared to the functional data, structural information concerning SGLT1 is relatively sparse. On the basis of hydropathy analysis of the primary amino acid
sequence and data relating to a set of glycosylation mutants (Turk et al. 1996), a secondary structure has been proposed 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. Mutagenesis experiments have identified certain residues critical to the proper trafficking of the protein to the plasma membrane (Lostao et al. 1995), and a chimera of SGLT1 and its low affinity isoform SGLT2 indicates that the glucose binding site may be localized to the C-terminal half of the protein (Panayotova-Heiermann et al. 1996). Recently, a truncation mutant of SGLT1 has shown that the essential structure for glucose binding and translocation can be provided by the terminal five helices of the transporter (Panayotova-Heiermann et al. 1997). There are however no experimental data regarding the specific location of residues that make up the sodium and/or glucose translocation pathway and confirmation of the proposed SGLT1 topology by standard techniques such as limited proteolysis, and construction of fusion proteins has yet to be carried out.

I report here the results of a cysteine scanning mutagenesis project of the region between putative transmembrane helices IV and V. I chose this region for analysis because hydropathy profiles of SGLT1 reveal that along with the residues that make up the adjacent putative transmembrane helices this region forms one of the longest hydrophobic stretches in the protein. Algorithms which predict transmembrane domains interestingly have difficulty defining where the two α-helices begin and end due to the high degree of hydrophobicity and helical propensity of the entire region. Without the geometry of the lipid bilayer limiting the length of transmembrane segments these
algorithms would designate the entire region as a single transmembrane α-helix. The breaking up of this hydrophobic stretch into two transmembrane segments, however, is given experimental support by the glycosylation of a mutant with an acceptor insertion in between positions 169 and 170 (Turk et al. 1996).

The effect of the systematic individual replacement of amino acid residues 162-173 inclusive was tested by expressing the resulting mutant transporters in the *Xenopus* oocyte. 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 MTSEA and MTSES. The results demonstrate that while none of the residues play an absolutely essential role in transport, some may be located in close proximity to the pathway that extracellular sodium takes to its binding site in SGLT1. In previous reports, one of the mutants, A166C has already been characterized in detail using the *Xenopus* oocyte system and the COS-7 cell system.

**Results and Discussion**

*Construction and verification of the mutants*

Each amino acid in the region in between putative transmembrane helices IV and V, residues 162 to 173, was replaced individually with cysteine in wt rabbit SGLT1 as described under Methods 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 the mutants between and including the BclII restriction enzyme sites was identical to wt.
Figure 1 Predicted topology of SGLT1 (Turk et al. 1996) showing the location of the single cysteine mutants.

**αMG induced Na⁺ currents mediated by the mutants**

The ability of the mutants to function as Na⁺/glucose cotransporters was assessed using the *Xenopus* 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 the application of increasing concentrations of αMG to the buffer perfusing the oocyte. In Figure 2, I show the results of such an experiment with an oocyte expressing the L173C mutant. When the functional expression is sufficiently high, the αMG induced currents as a function of αMG concentration can be fitted to the Michaelis-Menten relationship and an apparent $K_m$ calculated for the mutant transporter. From this kind of experiment, accurate determinations of an apparent αMG $K_m$ were made for the following eight mutants - F163T, A166C, I167C, F168C, Q170C, L171C, T172C and L173C (Table 1). It was found that for three of the mutants, F163T, A166C and T172C, the apparent affinities for αMG are significantly reduced; 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 to wt. For the remaining four mutants, I162C, I169C, G165C, and S164C, although αMG induced currents were observed, the levels of these currents were too small to allow for an accurate determination of an apparent αMG affinity. From titrations of αMG concentrations up to 10 mM αMG, the best I was able to accomplish for these four mutants was an estimate of an upper bound to the apparent αMG affinity (Table 1).

I mostly attribute the low levels of αMG induced currents exhibited by the four mutants cited above to low levels of expression caused by the particular cysteine substitution. Without high affinity antibodies to detect SGLT1 on Western blots I cannot definitively test this hypothesis, however, I was able to rule out the possibility that the low current levels were due to poorly performed cDNA injections and/or unhealthy oocytes. The low levels of αMG induced currents for oocytes injected with the four mutants were consistently low despite the fact that 1) oocytes from the same batch injected with wt or another mutant cDNA would demonstrate much larger currents and 2) the coinjection of cDNA encoding SEAP and the SEAP assay performed on the same or previous day would demonstrate that these same oocytes expressing poor αMG induced currents were expressing high levels of SEAP. The low levels of αMG induced currents are therefore likely a direct consequence of the cysteine mutation.

Since the low levels of αMG induced currents were accompanied by much reduced amounts of charge movements (see following section), I further suggest that the four mutants are expressing poor transport function because they are not being properly
Fig. 2A shows the currents induced by increasing concentrations of αMG in an oocyte expressing the L173C mutant (V = -150 mV to -10 mV, increments of 20 mV). The data is fit to the Michaelis-Menten relationship.

Figure 2B shows the voltage dependency of the αMG apparent $K_m$ for the F163C, A166C, Q170C, and L173C and compares them to wt SGLT1. The error bars are standard deviations (n ≥ 3).
<table>
<thead>
<tr>
<th>Mutant</th>
<th>αMG apparent $K_m$</th>
<th>Mutant</th>
<th>αMG apparent $K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I162C</td>
<td>&lt; 0.2</td>
<td>F168C</td>
<td>0.055 ± 0.004</td>
</tr>
<tr>
<td>F163C</td>
<td>0.580 ± 0.038</td>
<td>I169C</td>
<td>&lt; 0.2</td>
</tr>
<tr>
<td>S164C</td>
<td>&lt; 0.2</td>
<td>Q170C</td>
<td>0.110 ± 0.013</td>
</tr>
<tr>
<td>G165C</td>
<td>&lt; 0.2</td>
<td>L171C</td>
<td>0.070 ± 0.016</td>
</tr>
<tr>
<td>A166C</td>
<td>0.905 ± 0.057</td>
<td>T172C</td>
<td>0.35 ± 0.041</td>
</tr>
<tr>
<td>I167C</td>
<td>0.150 ± 0.011</td>
<td>L173C</td>
<td>0.126 ± 0.012</td>
</tr>
</tbody>
</table>

Table 1. Apparent $K_m$ (in mM) for αMG of the various single cysteine mutants at -50 mV. wt SGLT1 apparent $K_m$ is 0.150 ± 0.024 mM. Errors are standard deviations (n ≥ 3).

trafficked to the plasma membrane. The charge movements of wt SGLT1 have been correlated to plasma membrane expression levels by Wright et al (Zampighi et al. 1995), and it has been reported 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 never processed to the plasma membrane (Wright et al. 1996). Nevertheless, it remains to be experimentally discounted whether any of the four mutations, I162C, I169C, G165C, and S164C, eliminates both transport function and charge movements from a true functional standpoint and not just by causing a trafficking defect.
Charge movements

SGLT1 expressed in oocytes demonstrates transient currents in response to voltage jumps that have been attributed to the reorientation of the empty transporter in the membrane and the binding of Na+ to the outside facing conformation (Loo et al. 1993). These transient currents are eliminated by the non-transported competitive inhibitor phlorizin which "locks" SGLT1 in its Na+ bound outside facing conformation. The integral of these transient currents represents a charge movement with a membrane potential dependency that follows a typical Boltzmann distribution of the form,

\[ Q(V) = Q_{\text{max}}/[1 + \exp((V - V_{0.5})zF/RT)] - Q_{\text{hyp}} \]

where \( Q(V) \) equals the charge that has moved in response to a voltage jump from the hyperpolarizing limit to \( V \), \( Q_{\text{max}} \) corresponds to the maximal charge transfer, \( Q_{\text{hyp}} \) is \( Q \) at the hyperpolarizing limit, \( V_{0.5} \) is the voltage at which the charge movements are half completed, \( z \) is apparent valence, \( F \) is Faraday's constant, \( R \) is the gas constant, and \( T \) is temperature.

As a secondary assay for screening the function of the various cysteine mutants, I measured the charge movements specific to the mutant transporters by voltage jump experiments in the absence and presence of saturating phlorizin, and then fit these charge movements to the Boltzmann distribution. Table 2 summarizes the \( V_{0.5} \) determined for eight of the twelve cysteine mutants. Note that for F163C, A166C, F168C and L173C, the \( V_{0.5} \) is shifted towards more positive potentials, and for Q170C and L171C, the \( V_{0.5} \) is shifted towards more negative potentials. In general terms, shifts in the \( Q(V) \) relationship
along the voltage axis are likely 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 a conformational change that moves charges or dipoles on amino acid side chains through the membrane electric field (i.e. 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 such membrane potential dependent transitions.

Note that as with the αMG induced currents, the low expression levels precluded an accurate determination of these parameters for the remaining four mutants, I162C, I169C, G165C, and S164C, even though phlorizin inhibitable transient currents were clearly detectable in every case.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>(V_{0.5})</th>
<th>Mutant</th>
<th>(V_{0.5})</th>
</tr>
</thead>
<tbody>
<tr>
<td>I162C</td>
<td>n.d.</td>
<td>F168C</td>
<td>24.1 ± 4.41</td>
</tr>
<tr>
<td>F163C</td>
<td>21.94 ± 3.55</td>
<td>I169C</td>
<td>n.d.</td>
</tr>
<tr>
<td>S164C</td>
<td>n.d.</td>
<td>Q170C</td>
<td>-11.7 ± 1.24</td>
</tr>
<tr>
<td>G165C</td>
<td>n.d.</td>
<td>L171C</td>
<td>-13.3 ± 2.2</td>
</tr>
<tr>
<td>A166C</td>
<td>19.53 ± 0.76</td>
<td>T172C</td>
<td>3.05 ± 1.55</td>
</tr>
<tr>
<td>I167C</td>
<td>2.71 ± 3.40</td>
<td>L173C</td>
<td>23.6 ± 3.20</td>
</tr>
</tbody>
</table>

Table 2 showing the \(V_{0.5}\) values for the mutants obtained from fitting the Q vs V curves to the Boltzmann relationship. wt SGLT1 \(V_{0.5} = -2.54 \pm 0.7\) mV. Errors are standard deviations (n ≥ 3).
Sensitivity to MTSEA and/or MTSES inhibition

For the eight cysteine mutants which demonstrated significant αMG induced currents, I also tested whether the introduction of the cysteine would allow the sulphydryl reactive compounds, MTSEA or MTSES, to inhibit transporter function. Neither compound has any effect on either the steady-state transport kinetics or transient currents exhibited by wt SGLT1, and the experiments were carried out in hopes 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 such their own chemical modification would lead to an alteration in transporter function. The functional screen was to first add αMG to the buffer perfusing the oocyte and then, while measuring the αMG induced currents, add either 1 mM MTSEA or MTSES to the buffer. For wt, I167C, L171C and T172C there was no obvious inhibition of the αMG 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 equally dramatic and rapid inhibition with 1 mM MTSES but not 1 mM MTSEA. In Figure 3, the time course of MTS inhibition of currents mediated by F163C, A166C, Q170C and L173C is shown, illustrating that the reaction with the MTS reagents is complete within minutes.

The sensitivity to either MTSEA or MTSES inhibition introduced by the cysteine substitutions at positions 163, 166, 170 and 173 indicate that these positions likely 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 analysed by the Cys scanning mutagenesis project (aa162-173), may be an α helix with one face exposed to the extracellular aqueous environment. In Figure 4, I show an α helical wheel representation of this region that highlights those Cys mutants which were inhibited by either MTSEA or MTSES. Note that these Cys mutants all cluster along one face of the hypothetical helical arrangement.

The region consisting of amino acids 162-173, based on hydropathy profiles, is highly hydrophobic. I 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. The experimental basis for considering the region as an extracellular loop is the glycosylation of a mutant with an acceptor insertion inbetween positions 169 and 170 reported by Turk (Turk et al. 1996). 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.

The results of the functional screen focused attention on the following four mutants, F163C, A166C, Q170C, and L173C, and I proceeded to study the nature of the MTS inhibition on each of them in detail.
Figure 3. Time course of the MTSEA or MTSE inhibition of the single cysteine mutants. F163C, A166C, and L173C are inhibited by 1 mM MTSEA, while Q170C is inhibited by 1 mM MTSES.
sensitive to MTSEA inhibition

sensitive to MTSES inhibition

Figure 4  Helical wheel showing the positions where either 1 mM MTSEA or 1 mM MTSES dramatically inhibits transport activity.

F163C and A166C - As reported previously, I found that the effect of MTSEA on the steady-state kinetics of A166C was twofold, 1) a reduction in the turnover rate, and 2) a reduction in the apparent αMG affinity. I also found that the exposure to MTSEA altered the kinetics of the transient currents exhibited by A166C and had the effect of shifting the Q vs V curve to more negative potentials. When the F163C mutant was characterized in detail before and after MTSEA inhibition I found that a similar set of changes in transporter function had occurred. Although transport activity was too low to accurately determine the apparent αMG affinity post-MTSEA for the F163C mutant, I
could estimate that the turnover rate had been reduced. Furthermore, the transient current data shows an almost identical shift in the Q vs V curve belonging to F163C towards more negative potentials following MTSEA exposure (Figure 5), ΔV_{0.5} = 21.93 ± 3.90 mV (for A166C, ΔV_{0.5} = 17.98 ± 1.23 mV).

![Diagram showing Q normalized vs V (mV)](image)

**Figure 5.** Representative experiment showing the shift in the Q vs V curve towards more negative membrane potentials caused by a 5 min exposure to 1 mM MTSEA.

**L173C** - The inhibition of the αMG induced currents for L173C shown in Figure 3 when analysed more carefully revealed that the MTSEA effect on L173C was very different from the one seen with F163C and A166C. Figure 6 shows that inward currents measured in the absence of sugar are inhibited by 1 mM MTSEA in an oocyte expressing L173C. This was never observed for control oocytes, oocytes expressing wt, F163C or A166C. In fact, if the baseline currents did fluctuate for these oocytes it would always be
in the opposite direction since on occasion the MTSEA had the non-specific effect of inducing a small inward current. Therefore what appears to be an inhibition of the αMG induced currents of L173C in Figure 3, in reality, is a shifting of the baseline due to the MTSEA exposure. I have determined that the L173C mutant compared to wt, F163C and A166C exhibits a much larger Na⁺-leak that is inhibited by MTSEA exposure (Figure 7). In contrast, the Na⁺-leak currents mediated by wt, F163C and A166C are not affected by exposure to MTSEA.

In addition to the MTSEA effect on the Na⁺-leak is there any other effect on either the steady state or transient kinetics of the L173C mutant? With respect to the αMG induced currents, I have not observed any significant changes. The apparent αMG $K_m$ after MTSEA exposure is $0.111 \pm 0.009$ mM compared with $0.126 \pm 0.012$ mM, and the $I_{max}$ following MTSEA exposure appears unchanged when experimental errors are taken into account. The transient current measurements, however, demonstrate that the Q vs V curve for the L173C mutant like the F163C and A166C mutants is shifted to more negative potentials by the reaction with MTSEA. I note though that the shift is more subtle and the $ΔV_{a.e}$ is only $7.66 \pm 4.65$ mV ($n = 3$) compared to $-20$ mV for the other two mutants.

Q170C - 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 vs V curve to more negative potentials, and only a modest decrease ($< 20\%$) in the $Q_{max}$. The exposure to MTSES of Q170C, however,
resulted no statistically significant shift of the Q vs V curve, and a substantial decrease (~50%) in the Q\textsubscript{max}. In addition, the apparent αMG K\textsubscript{m} was unchanged by reaction to MTSES and the decrease in transport activity observed in Figure 3 can be attributed to a change in the I\textsubscript{max}. Finally, it can be demonstrated that MTSES causes both I\textsubscript{max} and Q\textsubscript{max} to decrease by approximately the same factor and so it can be concluded that the Q170C turnover rate is relatively unaffected by MTSES. To summarize, all the data supports the hypothesis that the MTSES effect on Q170C is simply the inactivation of a subset of the functional transporters.

In experiments where I pretreated the oocytes expressing Q170C with 1 mM MTSEA, I 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 ammonium group left there by the reaction has little effect on transporter function (Figure 9).
Figure 6. A representative experiment showing that the baseline currents (currents measured in the absence of sugar and phlorizin) in an oocyte expressing L173C are inhibited by exposure to 1 mM MTSEA. The time course of the inhibition is similar to the one observed for the αMG induced currents depicted in Figure 3.
Figure 7. (upper panel) The Na⁺-leak currents (currents inhibited by 0.2 mM phlorizin) normalized according to the $Q_{\text{max}}$ is shown for wt, A166C, and L173C. The Na⁺-leak / $Q_{\text{max}}$ 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 is significantly inhibited by the exposure to 1 mM MTSEA.
Figure 8. The Q vs V curve for Q170C before and after MTSES. The curves have been shifted such that Q = 0 at extreme hyperpolarizing potentials to aid in the comparison.

nagc_rabbit  IYLSILSLLLYI FTKISADIFS GAIIFIQLTLG LDLYVAIIIL
nagc_human   VYLSILSLLLYI FTKISADIFS GAIFINLALG LNYLAIFLL
nagc_pig     VYLSILSMLLYI FTKISADIFS GAIFITLALG LDLYLAIFLL
naaa_pig     IYLSILSLFICV ALRISSDIFS GAIFIKLALG LLDYLAIFSL
nanu_rabbit  LYLSTLISLYI FTKISVDMS GAVFIQQALG WNIYASVIAL
nagl_human   LYLSTLSTLYI FTKISVDMS GAVFIQQALG WNIYASVIAL
nami_canfa   VYFAASLILYYI FTKLSVDLYS GALFIQESLG WNLVVSIVIL
putp_ecoli   RIISAVLIIFF TIYCAGIVA GARLFESTFG MSYETALWAG
panf_ecoli   VVLASLSSLVAF VGAMTVQFIG GARLLETAAG IPYETGLLIF

Figure 9. Multiple sequence alignment of selected members of the Na⁺/solute symporter family (Reizer et al. 1994). Underlined are the residues that I have mutated into cysteines (162-172 in rabbit SGLT1), in bold are the residues that when mutated into cysteines result in a transporter that is sensitive to inhibition by either MTSEA or MTSEA.
Conclusions

1) The results indicate that the SGLT1 apparent affinity for sugar (such as aMG) is extremely sensitive to amino acid substitutions in the loop region between putative transmembrane helices IV and V. Of the 12 consecutive residues studied, seven when changed to a cysteine result in either a substantially higher or lower apparent affinity for aMG. I speculate that these changes in apparent $K_m$ are the consequence of changes in the binding of Na$^+$ to the transporter and not due to an alteration in the sugar binding site itself.

2) Four of the cysteine mutants were inhibited by reaction with either MTSEA or MTSES (but not both) whereas the wt SGLT1 transporter was unaffected by these water soluble sulphhydryl specific reagents. These four mutants cluster on the same face of an alpha helical structure and thus support the theoretical prediction that this loop has a high alpha helical propensity.

3) When transient currents were studied, it was found that the reaction of MTSEA with F163C and A166C caused a shift in the Q vs V relationship towards more negative membrane potentials. This could be interpreted as a reflection of changes in the rate constants describing Na$^+$ binding and/or the reorientation of the empty transporter from inside facing to outside facing. Together with the effect of MTSEA on the transporter turnover rate, this suggests that the Na$^+$ interaction with the transporter and/or transporter reorientation within the membrane are related to rate determining steps in the transport cycle of SGLT1.

4) The effect of MTSES on Q170C included a decrease in the amount of charge
transferred by the mutant transporter. This may be due to an inactivation of a subset of the transporters present. It is tempting to speculate that this may reflect the multimeric nature of SGLT1.

5) Finally, MTSEA was also able to inhibit L173C. This inhibition of steady state currents has been shown to be predominantly due to an effect on the Na\(^+\) leak currents, i.e. Na\(^+\) currents mediated by the transporter in the absence of sugar. L173C exhibits substantially larger (approximately five fold higher after normalization) leak currents than wt SGLT1, which is strong evidence that this region is critical in Na\(^+\) binding and/or coupling of Na\(^+\) binding to sugar transport.

Utilization of the energy inherent in the Na\(^+\) electrochemical gradient across cell membranes is one of the fundamental mechanisms which biological systems use to transport substances past the hydrophobic lipid bilayer barrier. The structural/function relationships which underlie this mechanism have so far remained elusive. By combining the approaches of site-directed mutagenesis and chemical modification to the study of SGLT1, a model system for Na\(^+\) coupled transport, I have been fortunate to identify a region of the protein which is likely involved Na\(^+\) binding and perhaps the coupling mechanism. In conclusion, I believe that continued characterization of this region and adjacent regions will begin to elucidate the details of Na\(^+\) coupled sugar transport and Na\(^+\) coupled transport in general.
IV

Further characterization of the transport kinetics of the A166C mutant of the high affinity Na⁺/glucose transporter (SGLT1)
In this chapter I present the results from a number of experiments designed to further characterize the A166C mutant of SGLT1. Chapter II reported that for the A166C mutant, 1) the apparent αMG affinity is lower compared to wt, 2) the turnover rate is reduced, 3) the Q vs V curve is shifted towards more positive potentials, 4) MTSEA is able to inhibit the transporter by further reducing the apparent αMG affinity and turnover rate, and 5) MTSEA alters the decay kinetics of the transient currents and shifts the Q vs V curve towards more negative potentials. I extend the results reported in Chapter II by 1) characterizing the Na$^+$ dependency of the αMG induced currents in detail, 2) making direct measurements of the transport stoichiometry before and after MTSEA treatment, 3) estimating phlorizin binding affinity from charge transfer data, and 4) applying kinetic models to explain the transient current data.

Na$^+$ dependency of the sugar induced Na$^+$ currents - The Hill function has been used by Wright et al to describe the Na$^+$ dependency of the sugar induced Na$^+$ currents mediated by SGLT family members (Parent et al. 1992a; Panayotova-Heiermann et al. 1995a; Hirayama et al. 1996). In the case of SGLT1, fitting with the Hill function has yielded a Hill coefficient for Na$^+$ close to two and independent of membrane potential. With SGLT2, the Hill coefficient determined was closer to one and also independent of membrane potential (Mackenzie et al. 1996b; Mackenzie et al. 1994). These findings were believed to support the hypothesis that the Na$^+$:glucose stoichiometry of SGLT1 is 2:1 and that of SGLT2 is 1:1. In addition to the Hill coefficient data, the 2:1 stoichiometry of SGLT1 has been confirmed by simultaneous measurement of Na$^+$
current and αMG flux (Lee et al. 1994) and also by measurement of the reversal potential as a function of sugar concentration (Chen et al. 1995). Similar experiments for SGLT2 have not yet appeared in the literature.

Recently, a chimera consisting of the N-terminal half of SGLT2 and the C-terminal half of SGLT1 was characterized. As part of the characterization, it was reported that the Hill coefficient for Na\(^+\) varied from 0.8 \(\pm\) 0.4 at -150 mV to 1.5 \(\pm\) 0.6 at -70 mV (Panayotova-Heiermann et al. 1996). Due to the low level of expression for this chimera, however, the fitting procedures yielded parameter estimates with errors too large to be certain whether or not the Hill coefficient was varying with membrane potential. Consequently, it was difficult to make any definitive conclusions about the Na\(^+\):glucose stoichiometry for this chimera.

When the Hill function is used to describe the Na\(^+\) dependency of the sugar induced Na\(^+\) currents mediated by the A166C SGLT1 mutant, the fitting procedures yield a Hill coefficient that varies with membrane potential (Figure 1A). At -150 mV, the Hill coefficient is 1.20 \(\pm\) 0.15 and increases progressively until at -10 mV, it is 2.11 \(\pm\) 0.18. The level of expression of the A166C mutant is high enough to ascertain that the variation in the Hill coefficient is necessary for the Hill function to fit the data. Fixing the Hill coefficient (n) at any value between 1 and 2 leads to an extremely poor description of the ensemble of I vs Na\(^+\) relationships for the given series of membrane potentials (i.e. -150 mV to -10 mV). To illustrate this point, Figure 1B shows the data corresponding to -150 mV in Figure 1 fit to a Hill function with n = 2 and n = 1.2.
Figure 1A. A representative experiment showing the Na\(^+\) dependency of inward currents induced by 1 mM αMG in an oocyte expressing the A166C mutant. The 8 sets of data correspond to measurements made at 8 membrane potentials (-150 to -10 mV, increments of 20 mV). The smooth curves are the Hill function fits to the data.

Figure 1B. The data corresponding to -150 mV in Figure 1 is shown fit to a Hill function with \(n = 2\) (dotted line) and \(n = 1.2\) (continuous line).
Although the Hill coefficient has generally been used to estimate the stoichiometry of an enzyme substrate interaction, I do not believe that the variation in the Hill coefficient with membrane potential indicates that the Na\(^+\)/glucose coupling ratio changes from 2 to 1 as the membrane potential is decreased from -10 mV to -150 mV. Such a hypothesis would involve a radical reworking of the kinetic models which so far have been more than adequate. Instead I find an explanation for the variation in the Hill coefficient with membrane potential by reexamining the appropriateness of applying the Hill function to the case of a Na\(^+\) coupled transporter with 2:1 stoichiometry. It can be shown that when the two Na\(^+\) binding events are considered separately instead of as a single transition, the Hill function cannot be expected to describe, in the most general case, the Na\(^+\) dependency of the rate of transport. Instead, the exact expression describing transport rate as a function of Na\(^+\) takes the following form,

\[
V_{\text{max}} \cdot \frac{[\text{Na}^+]^2}{([\text{Na}^+]^2 + b[\text{Na}^+] + c)}
\]  

(1)

where both b and c are functions of rate constants and \(V_{\text{max}}\) is the maximal rate of transport. Equation (1) reduces to the Hill function with \(n = 2\) for the case where \(b << 1\) and the Hill function with \(n = 1\) for the case where \(c << 1\).

A useful quantity, equivalent to the apparent affinity constant of the Hill function is the concentration at which transport is half maximal,

\[
K_{0.5} = \frac{b}{2} + \frac{\sqrt{b^2 + 4c}}{2}
\]  

(2)

I emphasize that equation (1) is able to describe the data in Figure 1 to exactly the
same "goodness of fit" as the Hill function (Figure 2). The values for $K_{0.5}$ obtained are the same as the apparent affinity constants obtained by fitting the Hill function. The essential difference is that equation (1) assumes that the $Na^+$:sugar coupling stoichiometry is 2:1 and that the two $Na^+$ binding events are described by two transitions.

Figure 2. The same data in Figure 1 is shown here fit to the equation $V_{\text{max}} \times [Na^+]^2 / ( [Na^+]^2 + b\times[Na^+] + c)$. For -150 mV, $V_{\text{max}} = -296 \pm 4.8$, $b = 9.04 \pm 0.87$, $c = 25.4 \pm 4.9$; for -10 mV, $V_{\text{max}} = -161 \pm 45$, $b = 0 \pm 37$, $c = 3161 \pm 396$.

The A166C is "well behaved" compared to wt - At this point I emphasize two observations about the A166C mutant that make an analysis of its kinetics more straightforward than for wt SGLT1.

1) In chapter 2 it was shown that the $Na^+$-leak currents (steady state currents inhabitable by phlorizin) are much smaller for the A166C mutant than for wt. Chen et al discuss the point that if the $Na^+$-leak current represents a cycling of the transporter in the absence of sugar, then measurements of the $\alpha$MG induced currents made by taking the
difference of the currents measured in the presence and absence of αMG may be an underestimate of the transport activity (Chen et al. 1995; Chen et al. 1997). For instance, at progressively higher αMG concentrations there will be a shifting away from the Na⁺-leak pathway because the probability of the Na⁺ pathway coupled to αMG transport is greater. Therefore, the subtraction of the currents measured in the absence of αMG may be subtracting more Na⁺-leak than is actually present when Na⁺ coupled αMG transport is taking place. The greater the leak, the greater the error, and hence the αMG induced currents for the A166C mutant may be better estimates of transport activity than those that have been made for wt.

2) In the subsequent section I observe that for wt SGLT1 there is an exponential-like decay in transport activity over the first minute of 1 mM αMG exposure (Figure 3, upper right panel). This decay has been reported by others in abstract form for oocytes and also LLCKP-1 cells (Bennett and Kimmich, 1996) and seems to exist in other Na⁺-coupled transporters such as the Na⁺/glutamate transporter (Zerangue and Kavanaugh, 1996). So far, no satisfactory explanation has been put forward to account for this decay in transport activity. Although the amount of inactivation of the transporter due to the decay can be somewhat variable, the results from a large number of experiments indicate that the decay correlates with the αMG concentration (the closer to saturating concentrations the faster the decay) but not with the absolute level of transport activity. Together with the fact that a new plateau is reached (the decay is exponential-like), this suggests that the decay cannot be due to the accumulation of substrates inside the oocyte and transinhibition effects. Perhaps the decay is analogous to the “inactivation” that has
been documented for the Na⁺/Ca²⁺ exchanger (Hilgemann et al. 1992).

The A166C mutant does not exhibit as pronounced a decay as wt. For instance at 10 mM αMG, the A166C mutant is at close to saturation, but there is no evidence of the same decay exhibited by wt over the first few minutes of αMG exposure (Figure 3, lower right panel). Granted that the A166C absolute current levels are smaller than the ones depicted for wt in this particular example, but the observation holds even when the absolute current levels are comparable. As a consequence, the measurement of the transport activity of the A166C mutant is more straightforward than for wt and in experiments where transport activity is measured for a series of substrate concentrations, one right after the other in a single oocyte, the results are not going to be "contaminated" by the decay phenomenon to the same extent.

When I carried out experiments to measure the Na⁺ dependency of transporter activity for wt at low αMG concentrations to minimize the effects of the Na⁺-leak and the αMG-induced decay, I obtained data that could not be fit properly with a Hill function with a constant Hill coefficient. I believe that data obtained at higher αMG concentrations is well described by the Hill function with a constant Hill coefficient of approximately two because of the errors introduced by the Na⁺-leak and the αMG-induced decay. Therefore, the conclusions about the stoichiometry and not lumping the two Na⁺ binding events together that were made for the A166C mutant, should be extended to wt SGLT1.

*Direct measurement of coupling stoichiometry* - Since certain features of the MTSEA
inhibition of the A166C mutant suggest that the effect may have been on Na\(^+\) binding to the transporter, I wanted to determine as accurately as possible the coupling stoichiometry for the transporter before and after exposure to MTSEA. Studying the transport activity as a function of Na\(^+\) concentration has been previously used to determine the Na\(^+\):glucose coupling ratio (Mackenzie et al. 1996b). However, the A166C mutant following exposure to MTSEA, even for oocytes expressing high levels of transport activity prior to inhibition, does not demonstrate high enough transport activity for such a measurement to be made. Moreover, the discussion in the previous section shows that the analysis of the Na\(^+\) dependency of the \(\alpha\)MG induced currents for the SGLT1 mutant is complicated and the use of the Hill function to estimate stoichiometry may not be very informative. Therefore, I decided to make direct measurements of the coupling stoichiometry by simultaneously measuring Na\(^+\) flux by electrophysiology and sugar flux by radiolabelled tracer uptake.

In Figure 3 I show the current traces recorded for a control oocyte and oocytes expressing either wt or the A166C mutant (\(V = -50 \text{ mV}\)). At approximately 200 s, \(^{14}\)C-\(\alpha\)MG is added to the buffer perfusing the oocytes, and for the ones expressing wt or A166C there is induction of an inward current. The current traces also show the washout of the \(\alpha\)MG several minutes later and the subsequent return to baseline. These current traces were integrated to give an estimate of the amount of charge transport (and presumably Na\(^+\) transport) that had taken place as a direct consequence of the presence of \(\alpha\)MG, and the oocytes were individually subjected to scintillation counting to determine the amount of \(\alpha\)MG that had accumulated over the same period. From this data the
coupling stoichiometry is calculated and Figure 4 shows the results from pooling the experiments. I find that wt, the A166C mutant before and after MTSEA inhibition all demonstrate a 2:1 Na⁺:αMG coupling stoichiometry. For the A166C mutant I experimented with 10 mM αMG (as well as 1 mM αMG), since I wanted to compare the A166C and wt at close to saturating sugar concentrations.

Figure 3. Representative current traces for experiments to measure directly the Na⁺:αMG coupling ratio. In each of the four cases depicted the holding potential was -50 mV.
Figure 4. The Na\(^+\)−αMG coupling ratio determined for wt, the A166C mutant (166C) and the A166C mutant after MTSEA exposure (166EA).

Estimating phlorizin binding affinity - Phlorizin is a competitive inhibitor of SGLT1 that reversibly binds to the sugar binding site and prevents Na\(^+\)/glucose transport. Because phlorizin is a non-transported substrate, the kinetics of the phlorizin binding to SGLT1 is simpler than the kinetics of sugar transport (Moran et al. 1988). Fewer states need to be evoked to model phlorizin binding kinetics than to model transport kinetics. For a number of reasons, including unacceptably high non-specific binding, phlorizin binding experiments with tritium labeled phlorizin have not been carried out on SGLT1 expressed in Xenopus oocytes (Weber et al. 1991) and the apparent phlorizin binding affinity has not been measured as it has been with native SGLT1 in brush border membrane vesicles. Instead, estimates of a phlorizin K\(_i\) have been made for SGLT1 in experiments where
phlorizin is titrated in the presence of varying concentrations of αMG (Lostao et al. 1994; Panayotova-Heiermann et al. 1995a). However, these experiments are problematic because of possible trans-inhibition effects and the as yet unexplained decay of transport activity in the presence of saturating sugar (see previous section). I believe that a better way to study the phlorizin binding to SGLT1 is to make use of the observation that phlorizin upon binding to the transporter "locks" it in a conformation that cannot contribute to the charge movements that occur in response to voltage jumps. By measuring the amount of charge movement inhibited by increasing concentrations of phlorizin, one can calculate an apparent phlorizin binding affinity without even worrying about non-specific binding since the charge movements themselves are specific to SGLT1.

Figure 5 shows the time constants obtained from transient currents inhibited by increasing concentrations of phlorizin (0.78 μM to 0.2 mM). The invariance of the time constants with respect to phlorizin concentration strongly argues that the following assumption is valid - that the states in which phlorizin is bound to the transporter do not contribute to the measured charge movements (or if they do, then the contribution is uniform for all the phlorizin bound states). Therefore, the degree to which the maximum amount of charge movement is immobilized by a given phlorizin concentration is a direct measure of the amount of phlorizin bound to the transporter. Figure 6 makes use of this conclusion and demonstrates how the apparent phlorizin affinity can be calculated.

Figure 7 shows a representative experiment that illustrates the apparent phlorizin binding affinities for wt, A166C before and after MTSEA exposure. The apparent
affinity is slightly higher for the A166C mutant than wt, but after MTSEA inhibition the affinity is dramatically reduced. Pooling data from several experiments (n ≥ 3), the apparent phlorizin $K_m$ is $1.38 \pm 0.18 \mu$M for wt, $0.90 \pm 0.13 \mu$M for A166C, and $3.70 \pm 0.39 \mu$M for A166C after MTSEA inhibition (errors are standard deviations).

Figure 5. Transient currents were measured in the presence of increasing concentrations of phlorizin (0.78 μM to 0.2 mM) and then subtracted from the transient currents measured in the absence of phlorizin for an oocyte expressing SGLT1. This series of phlorizin-inhibitable transient currents was then fitted to a single exponential function from 2.8 ms to 99 ms after the onset on the voltage jump. The time constants resulting from the fitting procedure are reported as a function of the magnitude of the voltage jump (from a holding potential of -50 mV).
Figure 6 (left panel) Transient currents were measured for a series of phlorizin concentrations (0.391 μM to 1 mM) in an oocyte expressing A166C and then subtracted from the transient currents measured in the absence of phlorizin. This series of phlorizin-inhibitable transient currents was then integrated to produce the set of Q vs V curves reported here. Each Q vs V curve was fit to the Boltzmann equation to obtain values for Q_max. (right panel) The Q_max values are then plotted as a function of phlorizin concentration and shown to obey a Michealis-Menten relationship.
Figure 7. Double reciprocal plot showing that the apparent phlorizin binding affinity for the A166C mutant is greater than wt, but that after MTSEA exposure the apparent affinity is lower than wt. The apparent affinity for wt, $1.58 \pm 0.03$ μM; for A166C, $0.85 \pm 0.02$ μM; for A166C after MTSEA exposure, $3.02 \pm 0.12$ μM.
Figure 8. The kinetic model proposed by Parent et al to explain the kinetics of SGLT1 (Parent et al. 1992b).

Transient currents - The transient currents have been hypothesized to result from transitions between states 1, 2, and 6 in the 6-state model by Parent et al (Parent et al. 1992b) (Figure 8). In general, this 3-state subset of the 6-state model predicts two relaxation time constants that are each functions of $k_{16}$, $k_{61}$, $k_{12}$, and $k_{21}$. However, if $k_{12}$, $k_{21} >> k_{16}$, $k_{61}$ then the two time constants can be approximated by simpler expressions, one involving $k_{16}$, $k_{61}$ and another involving $k_{12}$, $k_{21}$. On that basis, according to Parent et al, the experimental relaxation time constant of the transient currents is determined largely by $k_{16}$ and $k_{61}$ (Parent et al. 1992b). Owing to the limited speed of the two-electrode voltage clamp, the much shorter relaxation time constant associated with $k_{12}$ and $k_{21}$ may be difficult to resolve.

In such a context, the observed changes in the transients of 166C may be
explained by decreasing $k_{16}$ from 0.035 to 0.024 and increasing $k_{61}$ from 0.005 to 0.1255. These new values for $k_{16}$ and $k_{61}$ are derived from fitting the time constant vs. voltage curve to a two state model (Figure 9). Obviously, this is not entirely correct but it provides reasonably good estimates. A possible interpretation of the result is that the reorientation of the empty carrier has been affected by the alanine to cysteine substitution at position 166.

![Figure 9](image)

Figure 9. The time constants ($\tau$) were obtained by fitting the phlorizin-inhibitable transient currents to a single exponential function from 2.8 ms to 99 ms after the onset of the voltage jump. The $\tau$ vs $V$ curve depicted here is fit to an equation derived from Eyring rate theory for a two state system assuming symmetrical energy barriers.

*One or two time constants?* - Although the work published by Wright et al continues to report a single relaxation time constant for the current transients of wild type rabbit SGLT1 (Hazama et al. 1997), I have found that two time constants are required to fit my own data for the identical transporter. My ability to resolve a second shorter time
constant may be explained by noting that my voltage clamp may be slightly faster and that I have chosen to isolate the current transients from the capacitive currents by using phlorizin rather than by curve fitting. When transients are measured using a lower gain and hence a slower voltage clamp, I find as Wright et al do, namely that reasonably good fits may be obtained with a single time constant. Furthermore, the method of eliminating the capacitive currents by curve fitting used by Wright et al (Loo et al. 1993) would tend to subtract out any component that does not decay at a rate significantly longer than decay rate of the capacitive currents. The second shorter time constant that I have resolved fits this description, as it happens to be approximately the same order of magnitude as the time constant for the capacitive currents.

The two time constants for wt rSGLT1 that I have measured exhibit a voltage dependency that is similar to that seen in the two time constants reported by Panayotova-Heiermann et al for the D176A mutant (Panayotova-Heiermann et al. 1994) and the two time constants reported by Chen et al for wild type human SGLT1 (Chen et al. 1996). The longer of the two time constants increases from ~10 ms to ~35 ms as the membrane potential decreases from 90 mV to 10 mV whereupon it begins to plateau. In contrast, the shorter time constant remains relatively unchanged over the same range of membrane potentials. In the paper by Chen et al (Chen et al. 1996), both time constants are hypothesized to be associated with transitions involving the empty transporter since both time constants were observed in the absence of inside and outside Na⁺. The experiments were conducted using the cut open oocyte system which gave them control over intracellular Na⁺ concentration as well as a ~0.1 ms voltage clamp. Even with the ultrafast voltage clamp, however, Chen et al could not resolve the time constant
associated with the Na\(^+\) association/dissociation events although this third time constant was inferred from kinetic modeling of their data. In the paper by Panayotova-Heiermann et al (Panayotova-Heiermann et al. 1994), the appearance of the shorter time constant in terms of a kinetic model was not discussed. Since the 6-state model was used to explain other features of the 176A mutant, however, the implication was that the shorter time constant could be related to \(k_{12}\) and \(k_{21}\), the Na\(^+\) association/dissociation events.

Comparing rabbit SGLT1 and human SGLT1 is not a straightforward process. It has been established that the two isoforms demonstrate significant differences in a number of pre-steady state and steady-state parameters. Therefore the work of Chen et al does not necessarily extend to my observations made on rabbit SGLT1. Application of the cut open oocyte system to wild type rabbit SGLT1 is needed to clarify whether the shorter time constant that I have measured using the two-electrode voltage clamp on intact oocytes is associated with a transition of the empty transporter or the binding of Na\(^+\) to the outside facing transporter. This is an important point because if the shorter time constant is related to \(k_{12}\) and \(k_{21}\), then the absence of this second time constant in the transients exhibited by 166C would automatically imply that the cysteine substitution has affected Na\(^+\) binding and/or debinding.

*How can the apparent sugar \(K_m\) be explained?* - I hypothesize that the decrease in the apparent affinity constant for αMG is not due to a change in the sugar \(K_m\), in other words \(k_{23}\), \(k_{32}\) are unaffected. This is supported by the observation that while the apparent affinity for αMG is decreased almost 5 fold, the apparent affinity for phlorizin remains relatively unchanged. Phlorizin is a non-transported competitive inhibitor that shares a
common binding site with the transported sugar. In phorizin binding experiments on transfected COS-7 cells, the phlorizin affinity constants for 166C and wt SGLT1 were determined to be the same within experimental error. In oocytes, the phlorizin affinity constant estimated from the effect of phlorizin on charge transfer was actually slightly higher for A166C (0.85 μM) than for wt SGLT1 (1.58 μM). If the sugar binding site is altered by the cysteine mutation then both the apparent sugar affinity and apparent phlorizin affinity would be altered in the same direction i.e. both decreased or increased. Since this is not the case, it is necessary to explore other possible explanations for the lower apparent sugar affinity exhibited by the A166C mutant. The most plausible explanation is that the cysteine mutation has caused a change in sodium affinity, since a lower Kₐ for sodium binding to the outside facing transporter would in most cases lead to a lower apparent sugar affinity.

The suggestion that the cysteine substitution at position 166 causes a change in the kinetics of sodium binding to the outside facing transporter is consistent with the earlier conclusion that the same mutation causes a change in the kinetics of the reorientation of the empty transporter. In all the acceptable models proposed to explain the kinetics of Na+/glucose cotransport, the reorientation of the transporter is followed immediately by the binding of sodium. Hence the two transitions have long been connected kinetically. That the two transitions can be affected by a single amino acid substitution is evidence of a structural link, while consideration of the direction in which the rate constants have been changed by the mutation allows for speculation as to the nature of this structural link. Since kₑ and the sodium Kₑ are increased, it is possible that
the 166C mutation stabilizes an intermediate state in the transition from inside to outside facing and that in this state the Na⁺ binding site is less accessible.

*Does a lower K_d for sodium imply a lower apparent phlorizin affinity?* - The 6-state model has been extended by Wright et al to include the effect of phlorizin on the transporter by introducing a phlorizin bound state (Loo et al. 1993). In this extension of the model, phlorizin binds to the transporter following the binding of the two sodium ions and "locks" the transporter in its Na⁺ bound state. Under this kinetic scheme, a lower K_d for sodium would imply a lower apparent phlorizin affinity, although the extent by which the apparent affinity is lowered would, as well, depend on the sodium concentration. It is not obvious that a set of values for the various rate constants (keeping the K_d for sugar and phlorizin constant) could be found that would simulate the decrease in the apparent sugar affinity without decreasing the apparent phlorizin affinity. The 6-state model does not present a framework for a simple explanation of the observation that compared to wt the αMG apparent affinity is lower and the phlorizin apparent affinity is higher for the A166C mutant.

*Evidence in favor of the Na⁺/S/Na⁺ order of binding* – The detailed analysis of the Na⁺ dependency of the transport activity for A166C and wt SGLT1 forces one to conclude that the two sodium binding events are not identical (re: when the Hill function is used to fit the data, the Hill coefficient obtained varies with membrane potential). This claim is supported by several independent investigations which show that phlorizin binding to SGLT1 is dependent on both Na⁺ and the membrane potential, whereas phlorizin dissociation is Na⁺ dependent, but potential independent (Kimmich, 1990;
Aronson, 1978; Moran et al. 1988). The Na\(^+\) dependency of phlorizin dissociation is strong evidence that the binding of the second Na\(^+\) ion is not equivalent to the first and that in ordered binding models the second Na\(^+\) ion must debind before phlorizin is able to dissociate from the transporter.

An alternative to the 6-state model that does not assume that the two sodium binding events are identical, is the 8-state model by Kimmich et al. (Bennett and Kimmich, 1996) which states that the second Na\(^+\) ion binds following the binding of sugar (or phlorizin). Recently, an analysis of the kinetics of the Na\(^+\)-leak that is observed for SGLT1 expressed in oocytes has provided substantial evidence in favor of the 8-state model (Chen et al. 1997). The Na\(^+\)-leak was shown to vary as a function of [Na\(^+\)]\(_o\) in a Michaelian fashion and the Na\(^+\) affinity constant for the Na\(^+\)-leak was several times lower than that for cotransport. Together with the observation that the sugar concentration required to balance the Na\(^+\) leak is [Na\(^+\)]\(_o\) dependent, these results are inconsistent with the Na\(^+\)-leak occurring following the binding of two Na\(^+\) ions as predicted by the 6-state model. The results, however, are consistent with the Na\(^+\)-leak occurring after a single Na\(^+\) ion binds to the transporter as predicted by the 8-state model.

The 8-state model also has an advantage over the 6-state model in explaining the changes in the apparent affinities for αMG and phlorizin that are produced by the cysteine substitution at position 166. If a Na\(^+\) ion binds following αMG or phlorizin binding, then changes in its affinity can also affect the αMG or phlorizin apparent affinity. Since αMG binding results in transport but phlorizin binding does not, the assumption is that the $K_d$ of the second Na\(^+\) ion following αMG binding is different from
the one for the second Na$^+$ ion following phlorizin binding. The hypothesis is that for the
A166C mutant the two Na$^+$ binding events have been altered in such a way as to produce
a higher αMG apparent $K_m$ and a lower phlorizin apparent $K_m$. 
V

Localization of the extracellular Na⁺ pathway in the Na⁺/glucose cotransporter (SGLT1)
Introduction

The Na\(^+\)/glucose cotransporter (SGLT1) is a multspanning membrane protein that uses the Na\(^+\) electrochemical gradient to drive the transport of sugar across intestinal and renal brush border membranes (Hediger, et al. 1987; Wright et al. 1996; Hediger and Rhoads, 1994). It belongs to a family of Na\(^+\) coupled cotransporters and translocates two sodium ions and one sugar molecule in each normal transport cycle (Lee et al. 1994; Chen et al. 1995). In the absence of sugar substrate, SGLT1 exhibits transient currents in response to rapid changes in membrane potential (Parent et al. 1992a; Loo et al. 1993; Panayotova-Heiermann et al. 1994; Chen et al. 1996). These transient currents result from transitions that immediately precede the binding of sugar, namely the reorientation of the empty transporter within the membrane and the binding of sodium. I report here the characterization of a single cysteine mutant of SGLT1 (T156C) that exhibits novel transient currents in the presence of sugar, which are distinct from the ones measured in its absence. One can interpret these transients to be the result of an altered membrane potential dependent transition that immediately follows sugar binding. Supporting this hypothesis, are altered binding kinetics of the non-transported inhibitor phlorizin which reveal that the binding of the second sodium has been affected. Furthermore, the introduction of the cysteine at position 156 allows the sulphydryl reactive reagent, methanethiosulphonate ethylamine (MTSEA) (Akabas et al. 1992), to inhibit the transporter in a manner that is prevented by sodium dependent phlorizin binding. Taken together these results are consistent with a Na\(^+\):sugar:Na\(^+\) ordered binding scheme (Bennett and Kimmich, 1996; Chen et al. in press) and demonstrate that position 156 in SGLT1 can directly influence the access of the second sodium ion to its binding site.
Along with the recent results of other cysteine mutants in this region (Lo and Silverman, submitted; Vayro et al., submitted), this provides important structural localization of the sodium pathway in a sodium cotransporter.

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 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 (Sarkar and Sommer, 1990) 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. Harvesting of the Xenopus laevis oocytes, preparation of the oocytes by collagenase digestion and injection of the cDNA were preformed as previously described (Lo and Silverman, submitted).

Two electrode voltage clamp - In all experiments, the oocyte currents were measured with the two electrode voltage clamp technique (Hille, 1992). I used a Geneclamp 500 amplifier, Digidata 1200 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 that -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 uptake buffer
was 100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES-Tris base (pH 7.4).

Results and Discussion
Neither sugar induced Na⁺ currents nor transient currents exhibited by wt SGLT1 (wt) in response to voltage jumps are altered by even prolonged exposure to methanethiosulphonate ethylamine (MTSEA) (Lo and Silverman, submitted). However, when a cysteine is substituted into any one of three positions in the putative extracellular loop connecting transmembrane helices IV and V (Fig. 1E) (Turk et al. 1996), the resulting mutant SGLT1 becomes sensitive to inhibition by the cysteine reactive compound (Fig. 1A). Fig 1B summarizes how these three single cysteine mutants, F163C, A166C and L173C have the voltage dependency of their transient currents (represented by charge vs. voltage curves) shifted towards more positive membrane potentials compared to wild type. Reaction with MTSEA, and hence formation of a mixed disulphide with a positively charged amine group, is shown to shift these charge vs. voltage (Q vs. V) curves back towards more negative membrane potentials (Fig. 1C, Table 1).

When the cysteine mutations are combined to produce double and triple cysteine mutants, the number of cysteines correlates with the degree to which the Q vs. V curve shifts along the voltage axis (Fig. 1D, Table 1). For instance, the midpoint of the Q vs. V curve (V0.5) for the triple cysteine mutant is 37.8 ± 2.1 mV compared to -2.5 ± 0.7 mV for wt and shifts ~91 mV to -53.4 ± 0.9 mV following MTSEA exposure. This shift is larger than the shifts demonstrated by any of the double cysteine mutants and those shifts
in turn are larger than the ones recorded for any of the single cysteine mutants. One notes that reducing the extracellular Na\(^+\) concentration also has the effect of shifting the Q vs. V curve to more negative potentials (for wt, V\(_{0.5}\) is shifted \(\sim\)70 mV when [Na] is reduced from 100 mM to 10 mM). Therefore, I interpret the above results as due primarily to an

![MTSEA inhibition graph](image.png)

**Figure 1A** Time course of MTSEA inhibition of the 163C, 166C and 173C mutants expressed in *Xenopus laevis* oocytes. Shown is the normalized current induced by αMG at V = -50 mV (current measured using a two-electrode voltage clamp setup in the presence of αMG minus current measured in its absence). At the time indicated, 1 mM MTSEA is added to the perfusate. [αMG] = 1 mM for the 163C and 166C data, [αMG] = 0.1 mM for the 173C data (since the 173C mutant demonstrates a higher apparent affinity for αMG).
Figure 1B Schematic showing that the charge transfer, Q as a function of the test potential, V for 163C, 166C and 173C is shifted to more positive potentials compared to wild type SGLT1 (wt). The Q vs. V curve is generated by integrating the phlorizin sensitive current responses to a series of 100 ms voltage pulses. The holding potential, $V_h$ is -50 mV and prior to integration the current transients are baseline corrected using the mean current values obtained over the final few ms of the voltage pulse. The Q vs. V curves are fitted to the Boltzmann relationship, $Q = Q_{max}/[1 + \exp((V - V_{0.5})zF/RT)] - Q_{hyp}$, where $Q_{max}$ is the maximal charge transfer, $Q_{hyp}$ is Q at the hyperpolarizing limit, z is apparent valence, F is Faraday’s constant, R is the gas constant, and T is temperature. $V_{0.5}$ is the membrane potential at which $Q = Q_{max}/2$. For wt, $V_{0.5} = -2.54 \pm 0.70$ mV; 163C, $V_{0.5} = 21.94 \pm 3.41$ mV; 166C, $V_{0.5} = 19.53 \pm 0.95$ mV; 173C, $V_{0.5} = 23.6 \pm 3.20$ mV.
Figure 1C Schematic showing that MTSEA exposure shifts the Q vs. V curves of 163C, 166C and 173C towards more negative membrane potentials. For wt, $\Delta V_{0.5} = 0$ mV; 163C, $\Delta V_{0.5} = 21.93 \pm 3.90$ mV; 166C, $\Delta V_{0.5} = 17.98 \pm 1.23$ mV; 173C, $\Delta V_{0.5} = 7.66 \pm 4.65$ mV.
**Figure 1D** Q vs. V curves for the double and triple mutants before and after MTSEA exposure. For comparison purposes, the dashed line shows the Q vs. V curve for wt. The solid lines are the Boltzmann relationships fitted to the data collected before MTSEA exposure. The dotted lines are the Boltzmann relationships fitted to the data collected after a 5 min. exposure to 1 mM MTSEA. The error bars are standard deviations (n ≥ 3).
Figure 1E Proposed secondary structure for rabbit SGLT1 showing transmembrane helices IV and V and the extracellular region connecting them.

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>F163C</th>
<th>A166C</th>
<th>L173C</th>
<th>163C/166C</th>
<th>163C/173C</th>
<th>166C/173C</th>
<th>Triple</th>
</tr>
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<tbody>
<tr>
<td>$V_{0.5}$ (before MTSEA)</td>
<td>-2.54 ± 0.70</td>
<td>21.94 ± 3.41</td>
<td>19.53 ± 0.95</td>
<td>23.6 ± 3.20</td>
<td>16.8 ± 3.3</td>
<td>29.8 ± 3.2</td>
<td>33.8 ± 1.8</td>
<td>37.4 ± 1.9</td>
</tr>
<tr>
<td>$V_{0.5}$ (after MTSEA)</td>
<td>n/a -1.1 ± 1.9</td>
<td>-1.14 ± 0.79</td>
<td>16.2 ± 3.4</td>
<td>-26.8 ± 2.8</td>
<td>-32.8 ± 2.0</td>
<td>-26.5 ± 4.6</td>
<td>-53.0 ± 4.6</td>
<td></td>
</tr>
<tr>
<td>Δ$V_{0.5}$</td>
<td>0 21.93 ± 3.90</td>
<td>17.98 ± 1.23</td>
<td>7.66 ± 4.65</td>
<td>43.5 ± 3.1</td>
<td>62.6 ± 3.6</td>
<td>63.4 ± 2.8</td>
<td>91.0 ± 2.7</td>
<td></td>
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Table 1. $V_{0.5}$ before and after MTSEA exposure and Δ$V_{0.5}$ for F163C, A166C, L173C, the double mutants and triple mutant. The values are in mV and the errors are standard deviations (n ≥ 3).
affect on the Na⁺ binding affinity, with the electronegativity introduced by the cysteine substitutions increasing the affinity, and the positive charges introduced by exposure to MTSEA decreasing the affinity. This is consistent with the idea that positions 163, 166 and 173 are at the entrance to a Na⁺-pore region in the SGLT1 transporter.

Although the above data suggests that positions 163, 166 and 173 may be influencing sodium binding by being located near the sodium pathway, more direct evidence for the localization of the sodium pathway to this region was found with another single cysteine mutant, T156C. Like the other mutants, the T156C mutant exhibited a Q vs. V curve that was shifted towards more positive potentials (Fig. 2A). Unlike the other mutants which were inhibited within seconds of 1 mM MTSEA exposure, however, the T156C mutant required higher concentrations of MTSEA and minutes of exposure time, suggesting that access to position 156 may be limited. Furthermore, although MTSEA exposure clearly affects the transients of T156C (Fig. 2B), instead of a shifted Q vs. V curve, the major consequence of the exposure is a reduction in the maximum amount of charge transfer, Q_{max} (the Q_{max} for the F163C, A166C, L173C mutants is comparatively unchanged by MTSEA). Lastly, phlorizin (a non-transported competitive inhibitor) was seen to provide partial protection against the MTSEA inhibition of T156C, something not observed for the MTSEA inhibition of any of the other three single cysteine mutants (Fig. 2C and 2D).

The phlorizin protection observation is most consistent with the following hypotheses, either 1) phlorizin binding induces a conformation change that makes T156C less accessible to MTSEA or 2) phlorizin binding allows the binding of a second sodium
Figure 2A (upper panel) The 156C mutant exhibits transient currents. In an oocyte expressing 156C, current responses to 100 ms voltage pulses were recorded before and immediately after the addition of 2 mM phlorizin. The difference in these current responses are plotted as a series of 13 overlapping traces corresponding to the 13 voltage pulses ($V$, -150 mV to +90 mV in 20 mV increments, $V_h$ is -50 mV). (lower panel) The 156C transient currents are inhibited by MTSEA. The same oocyte was exposed to 2 mM MTSEA for 10 minutes and the protocol for measuring the transient currents was repeated.
Figure 2B The Q vs. V curves for the transient currents shown in Figure 2A. (left panel). The 156C Q vs. V curve before MTSEA. (right panel) The Q vs. V curve after MTSEA.

Figure 2C Currents induced by 20 mM αMG before and after MTSEA exposure in an oocyte expressing 156C.

Figure 2D Currents induced by 20 mM αMG before and after MTSEA exposure in the presence of 2 mM phlorizin in an oocyte expressing 156C. Also shown are the currents after a second exposure to MTSEA in the absence of phlorizin.
ion which prevents MTSEA from reacting with the cysteine. A third hypothesis, that T156C is located within the binding pocket for phlorizin, is unlikely when consideration is given to the observation that the protection is incomplete and there is still a 25-30% reduction in transporter activity despite the fact that at 2 mM phlorizin greater than 98% of the phlorizin binding sites are occupied. Moreover, it would be inconsistent with recent reports that the five terminal transmembrane helices form the sugar pathway in SGLT1 (Panayotova-Heiermann et al. 1996; Panayotova-Heiermann et al. 1997). Therefore, even though the phlorizin protection effect is a direct consequence of phlorizin binding to the transporter, it is probably not due to the phlorizin molecule itself blocking the access of the MTSEA to the cysteine. This hypothesis is substantiated by an examination of the steady state phlorizin binding kinetics of the T156C mutant and comparing them with wt and the A166C mutant.

The binding of phlorizin to SGLT1 prevents sodium from dissociating and hence eliminates the charge movements that give rise to the transient currents (Parent et al. 1992a; Loo et al. 1993; Panayotova-Heiermann et al. 1994; Chen et al. 1996). By measuring the amount of transient current eliminated as a function of phlorizin concentration, an estimate of the apparent affinity of the transporter for phlorizin can be made. Such estimates show that the T156C mutant has over an order of magnitude lower affinity for phlorizin than either wt or the A166C mutant. In Table 2, the phlorizin affinities are compared with the apparent affinities for αMG calculated from measurements of the αMG dependency of the transport rate. The discrepancy between the changes in phlorizin apparent affinity and sugar apparent affinity suggest that the
Table 2. Apparent αMG and phlorizin affinities exhibited by wt, 166C and 156C. The apparent αMG affinities were determined from the αMG dependency of the αMG induced Na⁺ currents measured in oocytes using the two electrode voltage clamp method. The apparent phlorizin affinities were determined from the amount of transient current eliminated as a function of phlorizin concentration. The errors are standard deviations (n ≥ 3).

Cysteine mutations are not directly affecting the phlorizin/sugar binding site. Rather, the changes in the apparent affinities are due to changes in other rate constants. Identifying possible candidates for rate constants that influence sugar apparent affinity is complicated due to the large number of states involved in transport. However, the situation for the apparent phlorizin affinity is much simpler since phlorizin is a non-transported inhibitor. In the conventional ordered binding model for phlorizin where a sodium ion must bind to the outside facing transporter (C₀) before phlorizin (P) is able to bind and a second sodium ion binds following phlorizin binding (Moran et al. 1988; Kimmich, 1990),

\[ C_0 \leftrightarrow C_0Na^+ \leftrightarrow C_0Na^+P \leftrightarrow C_0Na^+PNa^+ \]  
(scheme 1)

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>166C</th>
<th>156C</th>
</tr>
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<tbody>
<tr>
<td>αMG Kₘ (mM)</td>
<td>0.150 ± 0.024</td>
<td>0.871 ± 0.042</td>
<td>0.951 ± 0.060</td>
</tr>
<tr>
<td>Phlorizin Kₘ (μM)</td>
<td>1.385 ± 0.189</td>
<td>0.903 ± 0.134</td>
<td>27.87 ± 1.861</td>
</tr>
</tbody>
</table>
the expression for the apparent phlorizin affinity is as follows,

\[ K_{p, \text{apparent}} = K_p \times \left( \frac{K_{d1}}{[Na^+]} + 1 \right) \times \left( \frac{[Na^+]}{K_{d2} + 1} \right) \]

The dissociation constants for each of the sodium binding events (\(K_{d1}, K_{d2}\)) can in theory both influence the apparent phlorizin affinity (\(K_{p, \text{apparent}}\)). In this particular case though, changes in the \(K_d\) for the first sodium ion cannot account for the lower \(K_{p, \text{apparent}}\) of the 156C mutant. The holding potential for the experiments conducted to estimate phlorizin affinity was -50 mV which according to the Q vs. V curve is a membrane potential where > 95% of the transporters are in the outside facing Na\(^+\) bound state. This means that \(K_{d1}/[Na^+] << 1\) and that sets an upper limit to the influence that changes in \(K_{d1}\) can have on \(K_{p, \text{apparent}}\). Therefore, to explain the higher \(K_{p, \text{apparent}}\) of the T156C mutant without changing the \(K_p\), the dissociation constant of the second sodium ion must have changed. In fact, if \(K_{d2}\) is small then the dependence of \(K_{p, \text{apparent}}\) on \(K_{d2}\) is linear and the observed ~25 fold increase in \(K_{p, \text{apparent}}\) for the T156C mutant can be accounted for by a ~25 increase in \(K_{d2}\). Such a hypothesis would be consistent with the phlorizin protection data where the reactivity of the cysteine at 156 may be altered by the binding of the second sodium.

Analysis of transient kinetic data also confirms the hypothesis that the T156C mutant exhibits altered binding of the second sodium. Examination of the time course of charge transfer in response to voltage pulses for wt and A166C indicates that the amount of charge transferred (Q) as a function of time (t) always increases and decreases monotonically for depolarizing and hyperpolarizing voltage pulses, respectively. For the
A156C mutant the behavior of some of these Q vs. t curves is quite different. Although the same monotonically increasing/decreasing curves are observed for most of the voltage pulses, notably different observations are made when the magnitude of the depolarizing voltage pulse extends beyond +50 mV (Fig. 3). For +70 mV and +90 mV voltage pulses, the Q vs. t curves increase to a maximum by ~20 ms and then decrease pseudoexponentially to a steady state value that is up to 15% lower than the maximum. The degree to which the value of Q at steady state differs from the maximum correlates with both the phlorizin concentration and the magnitude of the voltage pulse, the more positive voltage pulses and/or the smaller phlorizin concentrations giving the greatest differences.

I believe that the atypical behavior of the Q vs. t curve for the T156C mutant is a consequence of the subtraction protocol for the transient currents and the debinding of phlorizin induced by strong depolarizations. The transient currents specific to the transporter are isolated from the non-specific capacitive currents by subtracting the measurements made in the absence of phlorizin with those made in its presence. In the case of the +70 mV and +90 mV voltage pulses, phlorizin debinding during the application of the voltage pulse leads to a small specific transient current that when applied to the subtraction protocol ultimately produces the negative slope in the Q vs. t curve. The magnitude of the difference between the maximum value attained by the curve and its steady state value therefore corresponds to the amount of phlorizin that had dissociated from the transporter during the voltage pulse. In wt and A166C no such phlorizin debinding takes place and the curves do not exhibit a negative slope. With the T156C mutant, however, strong depolarizing voltage pulses do cause phlorizin to debind.
until a new steady state equilibrium is reached and that is reflected in the altered behavior of the $Q$ vs. $t$ curve. The most plausible explanation for the voltage induced transient debinding of phlorizin again has to do with the binding of a second sodium ion. If the conventional assumption is made that the actual binding and debinding events of phlorizin are not voltage dependent (Kimmich, 1990), then only certain kinetic schemes explain the data. Such schemes must include a pathway for the binding of phlorizin followed by the binding of a second sodium ion (see scheme 1). The voltage induced transient debinding of phlorizin is then due to the membrane potential dependency of the binding/debinding of the second sodium ion. The observation of transient debinding is made for the T156C mutant but not for wt or A166C because the higher T156C $K_d$ for the second sodium shifts the voltage dependency into a range accessible to the voltage clamp experiment.

The T156C mutant exhibits a second piece of transient kinetic data that strongly suggests that the mutation has affected the binding of the second sodium. Unlike wt SGLT1, the transient currents of the T156C mutant are not eliminated by saturating concentrations of $\alpha$MG. Fig. 4A shows that in the presence of 20 mM $\alpha$MG there are transient currents specific to the T156C mutant. These transients are characterized by an entirely different Boltzmann relationship than those measured in the absence of 20 mM $\alpha$MG and their characteristic time constant shows a different dependence on voltage (Fig. 4B). Their existence can therefore not be easily attributed to those transitions that account for the transient currents measured in the absence of sugar, namely the reorientation of the empty transporter and the binding of the sodium prior to the binding of sugar. More
likely, these transients are due to transitions following the binding of sugar. Since the binding/debinding of sodium on the cytoplasmic side of the transporter has been determined by others to be weakly if at all membrane potential dependent (Bennett and Kimmich, 1996), that leaves 1) the binding of the second sodium ion to the outside of the transporter or 2) the reorientation of the fully loaded transporter as the only possibilities. Although there is no experimental evidence in the transients themselves to distinguish between the two possibilities, 1) is more consistent with the data presented previously concerning phlorizin binding, where there is no reorientation of the fully loaded transporter. In fact, a discussion similar to that applied to the transient phlorizin binding observation is appropriate here. Namely, a significantly lower on rate for the binding of the second sodium and hence higher $K_{d2}$ can account for the characteristics of these transients measured in the presence of saturating αMG concentrations.

An important point needs to be stated concerning the clear non-equivalence of the sodium binding event following phlorizin binding and the one following sugar binding. Although there is evidence that phlorizin is transiently debinding in response to strong membrane depolarizations, there is no transient current measured in the presence of saturating phlorizin with the same characteristics of that measured in the presence of saturating αMG. This indicates that the sodium that binds after phlorizin is likely traversing a relatively small proportion of the membrane electric field compared to the sodium that binds after sugar. In other words, though the two binding sites are possibly comprised of the same set of residues, their arrangements with respect to the membrane electric field are significantly different. It is intriguing to speculate that this may be key
in explaining why phlorizin is not transported.

In conclusion, I have constructed various cysteine mutants of SGLT1 that provide a structural localization of the Na\(^+\) pathway. I speculate that positions 163, 166 and 173 are located near the entrance of a pore for Na\(^+\) and that position 156 is deep within this pore (Fig 1E). The continued application of cysteine mutagenesis combined with chemical modification using methanethiosuphonate derivatives, such as MTSEA, to this region of SGLT1 is likely to provide information about important structure function relationships relating to Na\(^+\) binding and permeation.
Figure 3 A representative experiment showing the charge transfer, Q as a function of time exhibited by an oocyte expressing the 156C mutant. Current responses to 100 ms voltage pulses were measured immediately before and after the addition of increasing concentrations of phlorizin. Q is the integral of the baseline corrected (see fig.1B caption) difference of these two current responses. Since increasing concentrations of phlorizin led to increasing immobilization of charge transfer, I compare the various curves by expressing Q as a percentage of the maximum value attained. I show the data for the 90 mV, 70 mV, 50 mV and 30 mV voltage pulses (V₀ is -50 mV).
Figure 4A (left panel) Transient currents exhibited by 156C in the presence of saturating αMG. In an oocyte expressing 156C, current responses to 100 ms voltage pulses were measured in the presence of 20 mM αMG and then in the presence of 2 mM phlorizin (0 mM αMG). The baseline corrected difference in these current responses are plotted as a series of 13 overlapping traces corresponding to the 13 voltage pulses (V, -150 mV to +90 mV in 20 mV increments, V_h is -50 mV). Right panel shows the Q vs. V curve corresponding to these transients measured in the presence of 20 mM αMG and compares it with the one for the transients measured in the absence of αMG (same oocyte).

Figure 4B Time constants of the transients in the presence and absence of saturating αMG. The transient currents were fitted to a single exponential decay function and the time constant obtained is plotted as a function of the test potential, V. The error bars are standard deviations (n = 4).
VI-1. Comments on the Na\textsuperscript{+}-leak

Theoretically, the Na\textsuperscript{+}-leak is defined as the Na\textsuperscript{+} flux mediated by the transporter that is not directly coupled to a sugar flux. In the 6-state model by Parent et al (Parent \textit{et al.} 1992a), this is the transition between states [C2Na\textsuperscript{+}]	extsuperscript{-} and [C2Na\textsuperscript{+}]	extsuperscript{+}, and in a modification of the 8 state model by Kimmich et al (Kimmich and Randles, 1988), this is the transition between states [CNa\textsuperscript{+}]	extsuperscript{-} and [CNa\textsuperscript{+}]	extsuperscript{+}. Experimentally, the Na\textsuperscript{+}-leak has been defined in oocyte voltage clamp experiments as the phlorizin sensitive current measured in the absence of sugar (Parent \textit{et al.} 1992a; Chen \textit{et al.} 1995). In experiments with cut-open oocytes, Chen et al has been able to show that when there is a complete absence of sugar on either side of the membrane and [Na\textsuperscript{+}]	extsubscript{o} = [Na\textsuperscript{+}]	extsubscript{i} = 50 mM (identical buffer compositions inside and outside except for 1 mM EGTA in the intracellular solution) the phlorizin sensitive current is inwardly rectifying i.e. there is no outward current at positive potentials (Chen \textit{et al.} 1995). Furthermore, the average outward current at 0 mV under such conditions is not statistically different from zero which is consistent with the identity of the phlorizin sensitive current being a Na\textsuperscript{+} leak current.

In two electrode voltage clamp experiments on oocytes where there is no control over the intracellular ion/solute concentrations, the phlorizin sensitive current exhibits a different membrane potential dependence. In my own experiments on rabbit SGLT1 with [Na\textsuperscript{+}]	extsubscript{o} = 100 mM, the phlorizin sensitive current has a reversal potential of approximately -30 mV and becomes outward at more positive potentials (similar results were published by Chen et al for human SGLT1(Chen \textit{et al.} 1997)). This is inconsistent with the hypothesis that the phlorizin sensitive current is exclusively a Na\textsuperscript{+} leak current since that situation would necessarily imply a positive $V_r$ for [Na\textsuperscript{+}]	extsubscript{o} > [Na\textsuperscript{+}].
hypothesis that has been put forward to explain the outward phlorizin sensitive current at positive potentials is that there must exist some SGLT1 substrate inside the oocyte. The identity of such a substrate, however, has not been experimentally determined. One possibility, that intracellular glucose is responsible has been considered, but given the report that intracellular glucose is below 0.05 mM for the oocyte (242) and that the transporter has a very low affinity for intracellular sugar (25-50 mM), it seems unlikely that this outward current is a glucose coupled Na+ flux. For instance, Figure 4 of the paper by Chen et al (Chen et al. 1995) shows that for [αMG]o = 10 mM and [Na+]o = 70 mM the reversal potential of the phlorizin sensitive current is approximately -35 mV. Assuming that glucose and αMG are indistinguishable to the transporter (i.e. the kinetics of binding and transport are the same for the two sugars), then the expression for reversal potential derived by Chen et al using these values would predict an unreasonably high intracellular sodium, i.e. [Na+]i = 210 mM.

Besides sugar binding to the inward facing transporter leading to an outward Na+ coupled sugar flux, a more likely possibility is that in addition to a Na+-leak there are other ions being conducted across the membrane by SGLT1 as part of an uncoupled ion flux. One can imagine that an intracellular cation such as K+ has a finite probability of binding to the transporter and giving rise to an outward current or the possibility that an anion such as Cl- may be carried by SGLT1 across the membrane. Both possibilities could explain the negative reversal potential that has been observed for the phlorizin inhabitable current by making this current the sum of a Na+-leak and other additional leak currents. If these additional leak currents did exist, it is intriguing to ask whether these
non-Na⁺-leak currents are taking a similar pathway through the SGLT1 transporter protein as the Na⁺-leak current. Can the pathway for Na⁺ have channel-like properties and if so does the selectivity for certain cations partially break down when the transporter enters into such a conductive state?

I consider the Na⁺ leak exhibited by SGLT1 in the context of the emerging view of the cotransporter as a channel (DeFelice and Blakely, 1996; Su et al. 1996). In a number of neurotransmitter transporters, channel-like conducting states have been observed in addition to the primary conducting state that is coupled to solute flux. For instance, Na⁺ leak currents mediated by the Na⁺/GABA (GAT1) and serotonin transporters are believed to be comprised of single channel events (Mager et al. 1996; Cammack and Schwartz, 1996). Moreover, Cs & Li⁺ produce comparatively large currents that are not coupled to neurotransmitter transport in oocytes expressing either GAT1 or the serotonin transporter (Lester et al. 1996; Mager et al. 1996; Mager et al. 1994). It is believed that these ions permeate without requiring neurotransmitter binding and that the permeation may be channel-like. An important question to be answered, therefore, in light of the evidence from neurotransmitter transporter research, is whether the Na⁺-leak exhibited by SGLT1 is comprised of clusters of ions (as in a channel) rather than single ion events (as in a carrier).

What do the Na⁺-leak data for the various cysteine mutants tell us about the structure/function relationships in SGLT1? Figure 7 of chapter 3 and Figure 1 below illustrate the two most striking observations that have been made for the Na⁺-leak of the various cysteine mutants studied in this thesis. 173C exhibits a larger Na⁺-leak than wt that is reduced ~50% by reaction with MTSEA, while 156C exhibits a smaller Na⁺-leak
than wt that is increased several fold by reaction with MTSEA. The placement of the ethyl amine and hence positive charge at position 173, I believe, results in a repulsion of Na⁺ ions that are diffusing towards the Na⁺ binding site, and the Na⁺-leak is reduced because the probability of Na⁺ getting to its binding site is reduced. The increase in the Na⁺ leak resulting from the placement of the ethyl amine at position 156, I again believe results from an electrostatic repulsion between the positive charge on the ethyl amine and a Na⁺ ion. This time, however, the Na⁺ ion is already bound at the Na⁺ binding site and the repulsion increases the probability that the Na⁺ ion debinds and transverses the membrane electric field. The analogy is with the "multi-ion pore" that has been previously discussed with respect to ion channels. The hypothesis synthesizes information about the known Na⁺:glucose 2:1 stoichiometry of SGLT1, the channel-like nature of the Na⁺ leak, and kinetic data that places position 156C near the binding site of the second Na⁺ ion.

Figure 1. The phlorizin-inhibitable currents (0.2 mM phlorizin) exhibited by the 156C mutant before and after exposure to 2 mM MTSEA for 10 minutes. The currents are normalized to the Q max measured at the time the phlorizin-inhibitable currents are measured. Note that the Q max is reduced by ~50% by the MTSEA exposure.
VI-2. **The molecular mechanism underlying the SGLT1 transient currents**

The phlorizin-inhibitable transient currents that have been measured for SGLT1 expressed in *Xenopus* oocytes are capacitive, and therefore represent charge movements associated with one or more membrane potential dependent transitions. Important questions regarding the identity of these transitions and whether they are the only membrane potential dependent transitions in the transport cycle have not been answered definitively. In this section I reevaluate the answers to these questions that have been suggested by others based largely on simplifying assumptions and not direct experimental evidence.

Since 2 Na\(^+\) ions are transported per transport cycle, the principles of microscopic reversibility and charge conservation require that the sum of all the charge movements associated with the transitions that comprise a complete transport cycle be equal to 2 elementary charges. The kinetic modeling by Wright and colleagues assume that all of the charge movements are represented by the phlorizin-inhibitable transient currents, i.e. the transient currents, microscopically, are due to the translocation of an equivalent of 2 charges entirely across the membrane electric field (Parent et al. 1992b; Loo et al. 1993; Hazama et al. 1997). Their modeling further suggests that the transient currents are the result of two kinds of membrane potential dependent transitions that take place in the absence of sugar, 1) the binding/debinding of Na\(^+\), and 2) the reorientation of the empty transporter within the membrane. With these two assumptions, the simulations of the steady-state kinetic data using a 6-state model result in the partitioning of 70% of the charge movement to the reorientation of the empty transporter and the remaining 30% to the binding/debinding of external Na\(^+\) (Parent et al. 1992b). Based on my results with the
various cysteine mutants, I believe that the above assumptions and conclusions are not correct for the SGLT1 transporter. I suggest that a better working hypothesis is that the phlorizin-inhibitable transient currents do not represent all of the charge movements in a complete transport cycle and are, to a first approximation, due to simply the binding of Na$^+$. The most direct evidence that other membrane potential dependent transitions exist that are not accessible to the transporter in the absence of sugar, comes from experiments with the 156C mutant. These experiments show that the 156C mutant exhibits transient currents in the presence of saturating $\alpha$MG with kinetics distinct from the phlorizin-inhibitable transient currents. I believe that these “$\alpha$MG” transient currents likely result from either the binding of a second Na$^+$ ion following sugar binding or the reorientation of the fully loaded transporter. They are measurable for the 156C mutant but not for wt, because the mutation has “slowed” down certain rate constants. Note that the experimental evidence indicates that the mutation has not created membrane potential dependency in a previously non-membrane potential dependent transition, and so wt SGLT1 would also be expected to demonstrate these “$\alpha$MG” transients except on a time scale that is beyond the two-electrode voltage clamp. Therefore, the membrane potential dependent transitions accessible only in the presence of sugar, generally speaking, apply to SGLT1 and not just the 156C mutant. Consistent with this hypothesis is the work of Kimmich et al which develops an 8-state kinetic model for LLCPK-1 patch clamp experiments (Bennett and Kimmich, 1996), and the cut-open oocyte data for human SGLT1 reported by Chen et al (Chen et al. 1995). The paper by Chen et al estimates that
the phlorizin-inhibitable transient currents represent 1.35 of the 2 charges that are translocated during a full transport cycle.

The second point I would like to make is that the phlorizin-inhibitable transient currents are due primarily to the binding/debinding of Na⁺, and not due partly to that transition and partly to the reorientation of the empty transporter. This may seem at first to be wholly inconsistent with the results of the cut-open oocyte data by Chen et al (Chen et al. 1996). For that reason I discuss first why I think that inconsistency is not the case, before proceeding with my reasons for believing in this simplified explanation of the transient currents.

The report by Chen et al showing that transient currents can be measured in the bilateral absence of Na⁺ and glucose with the cut-open oocyte, can be taken as evidence that the transient currents measured in the presence of Na⁺ are partly made up of charge movements associated with the reorientation of the unloaded transporter (Chen et al. 1996). It is a straightforward interpretation that accounts for the observation that the two time constants that are required to describe the transient currents before and after the replacement of Na⁺ with N-methyl-D-glucamine demonstrate qualitatively very similar membrane potential dependencies. Nevertheless, the observation of transient currents in the absence of Na⁺ does not necessarily imply that these transients are due to simply the reorientation of the empty transporter. The data do not exclude the possibility that an ion other than sodium is participating in a similar binding event that is contributing to the charge movement. For instance, protons which have been shown to be capable of driving sugar transport through SGLT1 could be binding to the transporter in the absence of Na⁺ (Hirayama et al. 1997; Hirayama et al. 1994). Chen et al address this issue by noting that
the affinity for external protons is 0.4 μM (which corresponds to pH 6.3) and that the experiments were carried out at pH 8.0 where the proton concentration is ~2% of its Km (Chen et al. 1996). The proton Km, however, was calculated from experiments measuring the H+ dependency of the H+ leak using the two electrode voltage clamp setup where internal Na+ concentration is not zero. Moreover, the value of 0.4 μM corresponds to V = -77 mV and though unreported the proton K_m would be expected to decrease with more negative membrane potentials. Therefore, besides measuring the proton Km using the cut open oocyte technique in the bilateral absence of Na+, an important experiment that remains to be done is to measure the effect of H+ concentration on the Q vs. V curves. Until this is done, one should not necessarily accept the conclusion that the reorientation of the empty transporter contributes substantial charge movement.

An important reason for explaining that the transient currents are primarily due to Na+ binding/debinding is that the data can be described very well using a two-state system. This is especially the case with the 166C, 163C, and 156C mutants where the transient currents are fit extremely well with just a single exponential (in contrast to wt SGLT1). Moreover, these three mutants demonstrate a τ vs V relationship that is suggestive of a two-state system and a Q vs V relationship that follows a Boltzmann distribution almost exactly. Now at this point with the data above there is no way to distinguish between the following two possibilities, 1) that the two states in the two-state system are the outside/inside facing conformations of the empty transporter, and 2) that the two states are the Na+ bound/unbound states of the transporter. The key piece of evidence that distinguishes between the two possibilities, supporting the second and
ruling out the first, pertains to the Na$^+$ dependency of the Q vs V relationship.

Figure 2 shows the Q vs V relationship for the 166C mutant at 100 mM Na$^+$ and 10 mM Na$^+$. I see that the 10-fold reduction in external Na$^+$ results in the Q vs V curve shifting to more negative potentials by ~85 mV, while the apparent valence remains essentially unchanged. For a two-state system (see Chapter I-2-4.) where the two states are Na$^+$ bound/unbound,

$$V_{0.5} = RT/zF \ln \left( \frac{[\text{Na}^+]}{K_m} \right)$$  \hspace{1cm} (1)

where $K_m$ is the Na$^+$ affinity constant in the absence of membrane potential. Experimentally, since the apparent valence is unchanged,

$$\Delta V_{0.5} = RT/zF \ln \left( \frac{[\text{Na}^+]_1}{([\text{Na}^+]_2} \right)$$  \hspace{1cm} (2)

where [Na$^+$]$_1$ and [Na$^+$]$_2$ are the Na$^+$ concentrations before and after. When the two-state system interpretation is applied to the data, the internal consistency is relatively good. The apparent valence calculated from equation (2) using the experimentally determined $\Delta V_{0.5}$ in Figure 2 is 0.68 and the apparent valence obtained from fitting the Q vs V curves in Figure 2 to the Boltzmann relationship is 0.79. I take this internal consistency as lending support to the interpretation that most of the charge movement in the transient currents is due to Na$^+$ binding/debinding.

Within the context of explaining the transient currents as arising from Na$^+$ binding/debinding, the effect of MTSEA on the various cysteine mutants can be interpreted as an effect on the $K_m$ of equation (1). Referring again to Figure 2, the
MTSEA exposure had the effect of shifting the Q vs V curve to more negative potentials by ~23 mV. This indicates that the Na⁺ affinity of the 166C mutant following MTSEA exposure is reduced. If I then extend this interpretation to the results presented in Chapter V concerning the effect of MTSEA on the double and triple mutants, I see that the progressive placement of positive charges in this region of SGLT1 leads to a progressive lowering of the Na⁺ affinity. I therefore speculate that positions 163, 166 and 173 are situated in the Na⁺ pore of SGLT1, probably near the entrance, in such a way that positive charges at these positions can affect the diffusion of Na⁺ ions towards the Na⁺ binding site. Theoretical treatments that calculate the energy barrier presented to ions by the vestibule of a biological membrane channel indicate that changes in the surface charge distribution of the vestibule can dramatically effect the shape and height of the energy barrier (Hoyles et al. 1996; Peskoff and Bers, 1988). In the final analysis, I believe that the positive charges introduced by MTSEA into this region of SGLT1 perturb transporter function by having an effect on the surface charge distribution of the vestibule leading to the Na⁺ binding site of SGLT1. This strongly substantiates the major conclusion of this thesis, that the Na⁺ pore is formed in part by putative transmembrane helix IV and the extracellular loop connecting it to putative transmembrane helix V.
Figure 2. Transient currents were measured in the presence and absence of 0.2 mM phlorizin for the 166C mutant at 100 mM and 10 mM Na⁺ (Na⁺ was replaced with Choline). The 166C mutant was then exposed to 1 mM MTSEA and the phlorizin-inhibitable transient currents were recorded at 10 mM Na⁺. The phlorizin-inhibitable transient currents were integrated after Na⁺-leak subtraction to obtain Q. The $Q_{\text{normalized}} = (Q + Q_{\text{hyp}}) / Q_{\max}$ is plotted here as a function of membrane potential.
VII. Future directions

A number of directions for future work are suggested by the results that have been presented in this thesis. They fall naturally into the following three categories, 1) further characterization of the cysteine mutants that have been described, 2) continued cysteine mutagenesis to explore the rest of putative transmembrane helix IV and V, and 3) application of other MTS derivatives to the cysteine mutants to answer additional questions about structure/function.

Further characterization of the cysteine mutants - Important confirmation of some key results from the characterization of the A166C mutant expressed in Xenopus oocytes have already been carried out in our lab using the COS-7 cell system. The Xenopus oocyte is a heterologous expression system and, in general, it is important to confirm the results for any mammalian protein in a mammalian cell system. Specifically, the transfection and expression of SGLT1 and the A166C mutant in the COS-7 cells has allowed for phlorizin binding measurements to be made. These experiments have independently confirmed that the change in $V_{\text{max}}$ caused by the cysteine mutation is due to a change in turnover and not a lower level of expression and/or trafficking to the plasma membrane. The expression of A166C in tissue culture cells also paves the way for the analysis of the cysteine mutants using patch clamp techniques. Bennett et al have demonstrated that such techniques, when applied to study SGLT1, provide a means to not only control membrane potential, but also solute concentrations on both sides of the membrane (Bennett and Kimmich, 1996). Furthermore, the expression of the SGLT1 mutants in a cell line may prove to be a more convenient way to produce the quantities of protein
necessary for biochemical and various kinds of structural analysis.

The role of intracellular ions and substrates in the transport cycle of SGLT1 is an extremely important issue that cannot be adequately explored by the intact oocyte system. Models that describe an alternately accessible binding site, for instance, would tend to predict the existence of mutations and chemical modifications that single-handedly affect both the external and internal binding events. Only through experimental access to the intracellular space can such hypotheses be tested. Generally speaking, experiments studying the trans-inhibition of wt SGLT1 and mutants with altered transport kinetics, will help construct a more rigorous kinetic model for Na'/glucose cotransport. As mentioned above, patch clamp experiments provide a means of addressing the intracellular binding events as do the cut-open oocyte and macropatch techniques.

In addition to experiments with new and more sophisticated techniques, there are a great many experiments that remain to be done using the two-electrode voltage clamp. An obvious one is the characterization of the ability of Li⁺ and H⁺ to drive sugar transport in the various cysteine mutants before and after MTSEA/MTSES treatment. Any change in the ion specificity would be very strong evidence in favor of a particular position being located in the cation translocation pathway. Another important experiment would be to characterize the Na⁺ dependency of the transient currents for the various cysteine mutants including the double and triple mutants, since that may enable one to distinguish between the possible molecular mechanisms behind charge translocation. Performing concentration jump experiments similar to the ones reported for GAT1 (Mager et al. 1996) may also serve to clarify the nature Na⁺ binding and the molecular origin of membrane potential dependency. There are also opportunities to study the temperature
dependency of steady-state and transient kinetic parameters for the various mutants and to examine the sugar specificity and phlorizin (and phlorizin analogues) interaction systematically and carefully.

*Further cysteine scanning mutagenesis* - The results of the cysteine scanning mutagenesis from position 162 to 173 suggest that it would be worthwhile to continue the cysteine scanning mutagenesis towards the N-terminus from 162 and towards the C-terminus from 173. If the hypothesis is correct in stating that the region is an α-helical transmembrane domain that forms part of the pore lining the extracellular Na⁺ pathway, then residues towards the N-terminal region of putative transmembrane helix IV may be directly participating in the binding of Na⁺. Likewise, the adjacent helix V may also be contributing residues that play a similar role. It is interesting to note that the positions which produce the MTS sensitive cysteine mutants, 163, 166, 170 and 173, demonstrate a relative distribution that is shared by positions, 146, 149, 153 and 156 (Figure 1). These positions in the wt SGLT1 sequence are all occupied by residues with side chains that contribute hydroxyl groups that could potentially help bind a Na⁺ ion. The importance of these four residues in Na⁺ binding has already been partially established by the detailed characterization of the T156C mutant.
Using other MTS derivatives to probe structure function - A great many MTS derivatives are available in addition to the MTSEA and MTSES used in the experiments reported in this thesis. The flexibility of the chemistry behind the synthesis of MTS derivatives also makes it possible to custom design MTS reagents for specific purposes. MTS derivatives that form mixed disulphides with bulkier and more highly charged groups can be used to
explore in detail the effect of charge and steric hindrance in this region of SGLT1. The position of the charge introduced by MTSEA and MTSES has been manipulated by placing the “target” cysteine in different locations, but this positioning can be refined by testing a series of MTS reagents with varying spacer groups between the sulphur atom and the charge. Such experiments may be able to provide detailed information about the electrodynamics of the pathway that the Na⁺ ion traverses to its binding site.

A variety of MTS derivatives are also available to label the SGLT1 protein for a wide range of experiments that do not primarily involve electrophysiology. For instance, MTS derivatives can introduce fluorescent probes that can potentially provide information about conformational changes. Such experiments depend crucially on labeling specificity, however, and that has not yet been determined for even the MTSEA/MTSES experiments on the various cysteine mutants. I recall that the SGLT1 into which “target” cysteines were engineered contains endogenous cysteines which obviously could be source of non-specific labeling. I also note that for any expression system there will likely be other membrane proteins that contain cysteines that can react with MTS compounds. Solutions to these potential sources of non-specific labeling include the construction of a cysteineless mutant, the development of protocols to label the non-specific sites with non-fluorescent analogues, and purification of the labeled SGLT1 protein.

Towards the purification of a functionally active SGLT1 protein the use of MTS-biotin to biotinylate the cysteine mutants may turn out to be very important. Preliminary experiments have already shown that the A166C mutant can be biotinylated without
inhibiting αMG induced currents and with only a slight change in the transient currents (Figure 2). Figure 3 shows that this treatment with MTS-biotin fully protects the A166C mutant from inhibition with MTSEA indicating that reaction with the MTS-biotin at position 166 is complete. Experiments are now underway to determine whether the biotin at position 166 after such treatment will be useful in detecting and purifying the SGLT1 mutant.

![Figure 2](image1.png)

**Figure 2**

![Figure 3](image2.png)

**Figure 3**
Lastly, MTS derivatives may be used in crosslinking experiments to identify residues on other transmembrane helices or connecting loops that in the real life tertiary structure are in close proximity to the region that I have been exploring. Perhaps this approach may lead to important information about the arrangement of the transmembrane helices, and help construct a three dimensional structure for SGLT1. The lack of such 3D structural information is perhaps the one greatest obstacle to an understanding of the molecular mechanism behind Na+/glucose transport and coupled transport in general.
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