The transport mechanism of the human sodium/myo-inositol transporter 2 (SMIT2/SGLT6), a member of the LeuT structural family

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Sasseville LJ, Longpré JP, Wallendorff B, Lapointe JY. The transport mechanism of the human sodium/myo-inositol transporter 2 (SMIT2/SGLT6), a member of the LeuT structural family. Am J Physiol Cell Physiol 307: C431–C441, 2014. First published June 18, 2014; doi:10.1152/ajpcell.00054.2014.—The sodium/myo-inositol transporter 2 (SMIT2) is a member of the SLC5A gene family, which is believed to share the five-transmembrane segment inverted repeat of the LeuT structural family. The two-electrode voltage-clamp (TEVC) technique was used to measure the steady-state and the pre-steady-state currents mediated by human SMIT2 after expression in Xenopus laevis oocytes. Phlorizin is first shown to be a poor inhibitor of pre-steady-state currents for depolarizing voltage pulse. From an up to threefold difference between the apparent ON and OFF transferred charges during a voltage pulse, we also show that a fraction of the transient current recorded for very negative potentials is not a true pre-steady-state current coming from the cotransporter conformational changes. We suggest that this transient current comes from a time-dependent leak current that can reach large amplitudes when external Na⁺ concentration is reduced. A kinetic model was generated through a simulated annealing algorithm. This algorithm was used to identify the optimal connectivity among 19 different kinetic models and obtain the numerical values of the associated parameters. The proposed 5-state model includes cooperative binding of Na⁺ ions, strong apparent asymmetry of the energy barriers, a rate-limiting step that is likely associated with the translocation of the empty transporter, and a turnover rate of 21 s⁻¹. The proposed model is a proof of concept for a novel approach to kinetic modeling of electronegic transporters and allows insight into the transport mechanism of members of the LeuT structural family at the millisecond timescale.

Na-glucose cotransporters (SGLTs); LeuT structural family; kinetic modeling; cotransporter; myo-inositol

cotransporters are molecular machines inserted in biological membranes that use the electrochemical energy gradient of one molecule to transport a given substrate. SMIT2 (also known as SGLT6), the product of the SLC5A11 gene, has been identified as a Na-coupled myo-inositol transporter in our lab in 2002 (9). It is notably expressed in the intestinal epithelium and in proximal tubules, where it mediates apical transport of myo-inositol (MI) (1, 24). It has been shown that this 66-kDa protein (2) transports MI with an affinity constant (Km) of 120 µM in the presence of Na⁺ (Km: 13 mM) using a 2 Na⁺:1 MI stoichiometry (3). It can be inhibited by phlorizin (Pz) with an inhibitory constant (K) of 76 µM (9).

SMIT2 is a member of the Na-glucose cotransporter (SGLT) family (the SLC5A gene family) that, in humans, comprises 12 members involved in Na-coupled transport of sugars, iodide, monocarboxylates, and vitamins (36). These cotransporters as well as many others including neurotransmitter cotransporters are thought to operate according to the alternate access mechanism (36). Briefly, alternate access stipulates that cotransporters maintain a strict stoichiometric ratio between transported solutes by presenting their binding sites to each side of the membrane alternatively.

SMIT2 is also a putative member of the LeuT structural family. The crystallized bacterial homolog VSGLT (13) (23% sequence identity and 43% similarity with SMIT2) shares similar structure with a number of other crystallized proteins (LeuT, Adic, Mhp1, BetP, CaT, ApeT, and GadC) (13, 14, 16, 29, 33, 39, 41–43), which all present an inverted repeat of a series of five-transmembrane (TM) segments (21). The presence of the inverted repeat architecture is a strong support for the alternate access mechanism where the displacement of two broken helices is thought to provide access of the binding sites to one side of the membrane at a time. We recently used simulated annealing (SA) to determine the parameters of a kinetic model of SGLT1 which is capable of reproducing all pre-steady-state and steady-state currents recorded as a function of extracellular Na⁺ and glucose concentrations (26). SMIT2 constitutes a very interesting candidate to apply and extend our approach to kinetic modeling using SA. When expressed in oocyte, it exhibits large electrogenic signals (3, 9), a cooperative Na binding (Hill coefficient of 1.4), and a strongly inward rectifying behavior in all experimental conditions tested (9). On top of obtaining a detailed model for a second member of the SLC5A family, the present study will suggest the specific kinetic step(s) through which SMIT2 can harness the energy stored in the membrane potential. Also, it will be interesting to see whether the model will be consistent with the absence of SMIT2-mediated outward current even when intracellular Na and MI concentrations are raised.

MATERIALS AND METHODS

Oocytes preparation and injection. Oocytes were surgically removed from Xenopus laevis frogs and defolliculated as described previously (18). All manipulations (anesthesia, surgery, and euthanasia of the animals after the final collection of oocytes) were performed in accordance with Canadian guidelines and with the approval of the Université de Montréal ethics committee. One to three days after defolliculation, oocytes were injected with 46 nl of water containing mRNA (0.1 mg/ml) to obtain maximal protein expression of human SMIT2 (9). Oocytes were maintained in Barth’s solution [in mM: 90 NaCl, 3 KCl, 0.82 MgSO₄, 0.41 CaCl₂, 0.33 Ca(NO₃)₂, 5 HEPES, pH 7.6] supplemented with 5% horse serum, 2.5 mM Na pyruvate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin for 3–5 days postinjection before electrophysiological measurements were performed.

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Electrophysiology. The simplified saline solution used in electrophysiological experiments was composed of (in mM) 90 NaCl, 3 KCl, 0.82 MgCl₂, 0.74 CaCl₂, and 10 HEPES, and pH was adjusted to 7.6 with NaOH. N-methyl-D-glucamine (NMDG-Cl) was used to keep the solution osmolality constant whenever Na⁺ concentration ([Na⁺]) was reduced below 90 mM. MI and Pz were directly added to saline of 1–2 M. The bath current electrode and the reference electrode were Ag-AgCl pellets. The bath current electrode and the reference electrode were filled with 1 M KCl and had resistances of 1–2 MΩ. The bath current electrode and the reference electrode were filled with 1 M KCl and had resistances of 1–2 MΩ.

The oocytes were clamped to a resting membrane potential (V₀) of −50 mV, and three repetitions of Vₘ steps (by increments of 15 mV, 300 ms duration, no series resistance compensation used) were applied with an interval of 1.7 s between each step. Ninety-five percent of the command voltage was reached within 3–4 ms. Data were obtained with a sampling frequency of 10 kHz and were low-pass-filtered using a cutoff frequency of 1,000 Hz. The three repetitions were averaged for each experiment.

Kinetic modeling. The kinetic models designed for the cotransport mechanism are based on the transition state theory. The cotransport cycle is viewed as a series of stochastic transitions between stable states. Consecutive states are separated by energy barriers, whose heights depend on membrane potential whenever a charge moves across a fraction of the membrane electrical field during the transition. For example, the rate constants representing the binding (kₒ) and release (kᵦ) of a substrate molecule (S) can be described as follows (where “i” refers to a given conformational state of the cotransporter and j = i + 1):

\[ kₒ = kₒ[S]e^{\alpha_i Vm^{FRT}}, \quad kᵦ = kᵦ[S]e^{-(1-\alpha_i) Vm^{FRT}} \]

where kₒ[S] and kᵦ[S] are the first order forward and reverse rate constants between state “i” and “j” when Vᵦm = 0 mV, zᵢ is the valence of the mobile charge times the fraction of the membrane electrical field crossed during the transition, αᵢ represents the asymmetry of the energy barrier, Vᵦm is the membrane potential, [S] is the concentration of relevant substrate, and F, R, and T are Faraday constant, gas constant, and temperature. Kinetic parameters were evaluated following the SA algorithm and the direct fitting approach previously described by Longpré et al. (26). Briefly, measured pre-steady-state currents are directly fitted using the kinetic model predictions instead of fitting the parameters of a multiexponential function representing the pre-steady-state currents. Following the SA algorithm, a random set of parameters is first chosen and the cost function is calculated. The cost function represents the difference between the model prediction and the experimental data. In our case, the experimental data are composed of the measured experimental currents (including both the pre-steady-state and the steady-state portions) and the charge displacement (Qᵦm) when the membrane potential returns to the −50 mV resting potential (mean values obtained from n = 6 oocytes from 3 different donor frogs). The cost function was calculated as the sum of the squared difference between corresponding experimental and theoretical points. The weighting was devised through trial and error to give the same weight to each insert presented in Figs. 6 and 7. One parameter is then randomly chosen and modified within a pre-set range of possible values. The cost function is then calculated again. If the new parameter is associated with a decrease in the cost function, it is automatically kept. If the new parameter produces an increase in the cost function, it still has a chance of being kept according to a probability function equal to e⁻ΔCF, where ΔCF is the difference between the values of the cost functions of two subsequent trials, and T can be conceptually understood as the temperature of the system. Whenever “thermal equilibrium” is achieved (i.e., when, for a certain number of trials, the number of accepted parameter changes that make the cost function larger is equal to the number of changes that make it smaller), T is lowered. After ~10⁶ iterations, the algorithm has converged toward a set of parameters that minimize the cost function.

The SA algorithm was written in MATLAB language (MathWorks, Natick, MA). Computations were made on the supercomputer Cottos from Université de Montréal.

With respect to the SA algorithm that was previously described (26), two modifications were implemented. First, when a parameter is changed to a new value, the micromovability [i.e., \( k_{ij_{1 \rightarrow 10}} = k_{ij_{10 \rightarrow 1}} \) and the transport stoichiometry (i.e., the complete transport cycle must carry two positive charges per MI transported) are respected by simultaneously modifying the value of all the other parameters (the kᵦ, kₒ, and zᵢ) using a balanced redistribution approach. Second, the rate constant (i.e., kᵦ, kₒ) space is explored following a logarithmic distribution of the parameter values to allow equal sampling within the different orders of magnitude allowed for each parameter. The rate constants were selected from a range going from 1 to 10⁵ s⁻¹ for substrate-independent steps in agreement with the millisecond time resolution of the TEVC technique. The selection ranges used for substrate-dependent steps were adjusted to take into account substrate concentrations in the millimolar range. For each connectivity, the equivalent moving charge, was randomly picked within a range going from 1.5 to 1.5 elementary charge.

Systematic approach to determine optimal connectivity. On the basis of the success of our previous kinetic modeling study (26), a more systematic approach was used to determine the optimal connectivity and its associated parameter set. The connectivity defines the number of states and the sequence in which conformational changes and substrate binding reactions are organized to produce a complete cotransport cycle. Taking full advantage of the computational resources available through cluster computing, a number of predefined models with different connectivities were first generated and used to fit the whole experimental data set. For practical reasons, as the computer server used had an 8 CPU per node architecture with a computer time limit of 168 h per submission, the procedure to obtain the optimized parameter set for each model was run at least 8 times for 168 h each. Models that did not achieve satisfactory fit within this time limit were discarded. States and associated transitions were then removed from the successful models to simplify the connectivity as much as possible until it failed to adequately account for the experimental data set. Following this scheme, a total of 19 different connectivities were tested (see Fig. 5). As it was shown that SGLT1 could be properly modeled using a 7-state model with symmetrical energy barriers (all αᵢ being set to 0.5) (26), each connectivity was asserted both with variable αᵢ and with αᵢ set to 0.5. Thus, a total of 38 “models” were actually tested.

RESULTS

Incomplete inhibition of pre-steady-state current by phlorizin. The use of a reliable inhibitor is very useful in identifying the current component specifically mediated by a given transporter. Phlorizin (Pz) is the common inhibitor for SGLT cotransporters (10), including SMIT1 (18) and SMIT2 (3, 9) and it is known that Pz inhibition is dependent on both the external Na⁺ concentration and Vᵦm (38). Before applying the SA algorithm to determine the numerous kinetic parameters of any given model, it was important to check if Pz could be considered as an efficient inhibitor of the cotransporter when used at a concentration of 500 μM, which is close to its solubility limit in saline solutions. Figure 1A shows typical OFF currents measured for an oocyte expressing SMIT2 when Vᵦm is returned to the holding potential of −50 mV following a 300-ms pulse to +40 or −155 mV. These currents are shown for three conditions: 90
mM Na$^+$ (black line), 90 mM Na$^+$ with 500 μM Pz (light gray line), and 0 Na$^+$ (dark gray line). In the presence of 90 mM Na$^+$, the transient current recorded reflects both a fast capacitive current (in the first 3–4 ms) and a slow pre-steady-state component that can be inhibited by 500 μM Pz. In the presence of 0 mM Na$^+$, no slow pre-steady-state current can be observed and the current recorded is indistinguishable from the current recorded with 90 mM Na$^+$ and Pz. When $V_m$ returns from +40 mV, the transient current in the absence of external Na$^+$ (0 Na$^+$) shows only the capacitive current of the cell and, in the presence of 90 mM Na$^+$, a significant slow pre-steady-state current can be recorded. Surprisingly, in these conditions, adding 500 μM Pz to 90 mM Na$^+$ did not inhibit the pre-steady-state current and the 90 mM Na$^+$ + Pz current trace almost superimposed with the 90 mM Na$^+$ trace.

This clearly shows that Pz is a poor inhibitor of pre-steady-state current following a depolarizing voltage pulse. In the absence of external Na$^+$, the transferred charge associated with the OFF currents is shown to follow a strictly linear dependence on $V_m$, strongly suggesting a purely capacitive nature (see Fig. 1B). The transferred charge in the presence of 90 mM Na$^+$ and Pz clearly shows that Pz inhibition is compromised at $V_m$ more positive than −5 mV where it cannot be used to identify a SMIT2-specific current. On the other hand, as the transient currents in the absence of Na$^+$ closely represent the capacitive current of the oocyte, we used it to correct the transient current recorded in the presence of various Na$^+$ and MI concentrations to obtain the true time course of SMIT2-associated currents.

$Q_{on} \neq Q_{off}$. As Pz appears to be efficient in blocking SMIT2 in a membrane voltage range going from −155 to −5 mV, (see Fig. 1B), we use it to understand the exact nature of the SMIT2-mediated transient currents. An example of ON and OFF Pz-sensitive transient currents is presented in Fig. 2. In the presence of 90 mM Na$^+$, these currents are characterized by an extremely slow component ($\tau = 98 \pm 1$ ms, means ± SE, $n = 4$) at strongly hyperpolarizing potential. This slow component appears only at $V_m$ more negative than −110 mV, and the equivalent of the charge transported during the negative voltage pulse ($Q_{on}$) is not present in the off transient current when $V_m$ returns to −50 mV ($Q_{off}$) (see Fig. 2, A and B). Furthermore, such a slow component is not seen in the total current measured in the absence of Na$^+$, in the presence of Pz, and in noninjected oocytes (data not shown), indicating that this slow component is clearly mediated by SMIT2.

Fig. 1. Incomplete inhibition produced by phlorizin (Pz). A: typical OFF currents for a sodium/myo-inositol transporter 2 (SMIT2)-expressing oocyte. Currents measured when the holding potential is returned to −50 mV following a 300-ms pulse to +40 or −155 mV are shown for 90 mM Na$^+$ (black line), 90 mM Na$^+$ with 500 μM Pz (light gray line), and 0 Na$^+$ (gray line). If Pz inhibits well the OFF current from −155 mV, it is a poor inhibitor of the OFF current from +40 mV. B: transferred charges associated with the OFF currents at 0 Na$^+$ (black) and 90 mM Na$^+$ (gray) and 90 mM Na$^+$ with 500 μM Pz (light gray) are shown (paired measurements, means ± SE for $n = 4$ oocytes). The strictly linear dependence of the black curve with respect to membrane potential ($V_m$) suggests that the transient currents in the absence of Na$^+$ represent the capacitive current of the oocytes. In the presence of Pz, the transient current is close to the oocyte capacitive current from −155 to −20 mV, but for more positive $V_m$, the effect of Pz is progressively compromised.

Fig. 2. Inequality between the apparent transferred charges $Q_{on}$ and $Q_{off}$. A: typical phlorizin-sensitive currents (I$_{Pz}$) of SMIT2-expressing oocytes in the presence of 90 mM Na$^+$. Currents are presented for voltage steps from −50 to various $V_m$ presented in 30-mV increments. B: apparent $Q_{on}$ and $Q_{off}$ (area under the $I_{Pz}$-$V_m$ curve) associated with Pz-sensitive currents of SMIT2-expressing oocytes (means ± SE, $n = 4$).
It is possible to calculate the apparent transferred charges associated with SMIT2-mediated transient currents [i.e., the area under the transient portion of the phlorizin-sensitive current ($I_{Pz}$)-$t$ curves shown in Fig. 2A]. For very negative $V_m$, this clearly shows that the apparent ON transferred charges ($Q_{on}$) are up to three times larger than the apparent OFF transferred charges ($Q_{off}$) (see Fig. 2B). As the charges transferred do not come back when the potential returns from very negative $V_m$ to $-50\text{ mV}$, this cannot be due to a displacement current associated to the cotransporter conformational changes. In consequence, we have to consider this slow transient current as a SMIT2-mediated current in the absence of substrate (MI), i.e., a time-dependent leak current.

**Leak current characterization.** The steady-state leak current is defined as the Pz-sensitive current at a given Na$^+$ concentration in the absence of MI ($I_{Pz} = I_{Na} - I_{Na+Pz}$). The apparent leak current associated with SMIT2, as measured at the end of a 300-ms pulse, is shown in Fig. 3A. Examination of Fig. 3A reveals that, at hyperpolarizing potential, apparent leak currents at low Na$^+$ concentrations are much stronger than at 90 mM Na$^+$ concentration. This is in sharp contrast with leak currents found in SGLT1, where leak currents were stronger at higher Na$^+$ concentration as Na$^+$ is one of the cations mediating the leak current (7, 26). The time courses of the currents used to evaluate the leak currents in 90 and 0 mM Na$^+$ are shown in Fig. 3B for a voltage pulse from $-50\text{ mV}$ to $-155\text{ mV}$. In the absence of Na$^+$, the oocyte current stabilizes to a very negative level right after the end of the capacitive current. As reported above, in the presence of 90 mM Na$^+$, the current experiences a slow decay with an average time constant of $98 \pm 1\text{ ms}$. In the presence of Pz (with either 10 or 90 mM Na$^+$), the currents reach a stable value right after the capacitive current is over (i.e., within 5 ms). This shows, in agreement with the conclusion reached above, that the slow decay observed in the presence of 90 mM Na$^+$ is completely Pz-sensitive and, thus, must be mediated by SMIT2. The stable amplitude reached by the leak current in 90 mM Na$^+$ at the end of the voltage pulse is consistent with the previously reported characterization of SMIT2’s leak current (9) where the Na$^+$ concentration dependency was not explicitly examined. Considering the steady-state leak current presented in Fig. 3A, it can be estimated that the leak current at very negative $V_m$ is inhibited by external Na$^+$ with an apparent affinity constant in the order of $\sim 10\text{ mM}$. This is comparable to the affinity constant published for SMIT2 Na/MI transport at very negative $V_m$ [e.g. 3 mM (9)]. These results show that a large leak current exists through SMIT2 in the absence of Na$^+$. In the presence of Na$^+$, it is conceivable that a negative membrane potential progressively brings the cotransporter in a Na-bound conformation which would be less leaky than the Na-free conformations. This would explain why the leak current slowly goes down to a low value under these circumstances.

**Extraction of pre-steady-state, SMIT2-mediated currents.** To obtain the most reliable set of SMIT2-mediated currents which would exclude the time-dependent leak current, the following strategy was used.

First, Pz was not used to obtain pure SMIT2 currents because it was shown to be poorly effective for $V_m$ more positive than $-5\text{ mV}$ (see Fig. 1B). The oocyte capacitive current was removed by subtracting the transient portion of the currents recorded in 0 Na$^+$ from the currents recorded in presence of various concentrations of Na$^+$ and MI. This is appropriate as the curve of the charge displaced vs. $V_m$ is linear in the absence of Na$^+$ and matches the charge displacement recorded in the presence of Pz within the voltage range where Pz is effective (see Fig. 1B). Second, we needed to obtain SMIT2-specific transient currents that reflect the pre-steady-state charge displacement. In the case of SGLT1, we have previously shown (25) that the leak current is going through the cotransporter (as it is Pz-sensitive) using a channel-like pathway that does not require going through the different steps that lead to cotransport. In this view, the leak current runs in “parallel” with cotransport current and is not involved in the cotransport mechanism per se. To avoid the time-dependent leak current of SMIT2, we excluded from our measurements the currents recorded at membrane potentials more negative than $-110\text{ mV}$ (see Fig. 2A). In addition, as the steady-state current (measured at the end of the voltage pulse) is systematically subtracted from the recordings in the process of obtaining the transient currents mediated by SMIT2, the steady-state leak current is not present in the data used to test the different kinetic models. In the presence of various MI concentrations, the steady-state current recorded prior to

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**Fig. 3. Na$^+$ dependence of the leak current mediated by SMIT2.** A: the steady-state phlorizin-sensitive currents ($I_{Pz}$) measured in the absence of myo-inositol are shown as a function of the indicated external Na$^+$ concentrations (paired measurements, means $\pm$ SE, for $n = 4$). B: time courses of the currents used to obtain $I_{Pz}$ in the presence of 90 mM Na$^+$, 0 mM Na$^+$, and 90 mM Na$^+$+Pz are shown for a voltage pulse from $-50$ to $-155\text{ mV}$. Current traces were aligned to start from an identical level at $V_m = -50\text{ mV}$. 

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adding MI was subtracted in such a way that the steady-state current reached reflects exclusively the Na/MI cotransport current.

Explicitly, the comprehensive data set that we have used is composed of the transient currents in the presence of 0, 10, 20, 40, and 90 mM Na⁺ in the absence of MI, and the transient plus steady-state currents in the presence of 90 mM Na⁺ and 0.02, 0.05, 0.1, 0.25, and 1 mM MI, for voltage pulses ranging from −110 to +40 mV (see Fig. 4). These currents were averaged for n = 6 oocytes from three different donor frogs. Individual oocyte expression levels were normalized on the basis of the amplitude of MI-dependent steady-state current. The resulting currents are shown in Fig. 4 as gray lines whose widths correspond to the SE of the normalized currents. In many instances, the gray lines corresponding to the experimental data are not visible because they superpose with the model predictions. In Fig. 4, gray boxes are used to indicate the time domain where the experimental data were fitted. In our hands, voltage steps resulting from TEVC apparatus reach 90% of the command potential only after 3 ms and this could vary during an experiment. As long as the voltage is not completely settled, the time course of the currents cannot be accurately measured and we chose not to use the first 3–4 ms after a Vᵐ change. Nevertheless, the transferred charges associated with those first few milliseconds [the areas under the I(t) curves] are totally valid. Thus, the first few milliseconds of ON and OFF voltage steps were discarded from the fitted I(t) curves but the associated OFF transferred charges were measured during the entire duration of the voltage pulses.

**Determination of the optimal kinetic model using SA algorithm.** The optimal connectivity for reproducing the experimental data set was determined following the general procedure described in MATERIALS AND METHODS. Explicitly, the procedure started with an overdefined model, i.e., purposely presenting more states than required for reproducing the complete data set. As hSGLT1 electrogenic currents could be successfully described using a 7-state model (26), we initially tested four 8-state models as shown in Fig. 5, a–d.

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**Fig. 4.** Experimental data. Means of normalized experimental currents are shown as gray lines whose widths correspond to the calculated SE, with n = 6 oocytes from 3 different donor frogs. Calculated currents based on model p shown in Fig. 5 are compared with the currents measured in the presence of various concentration of Na⁺ (A–E) and various concentrations of myo-inositol (MI; F–J). Voltage pulses of −110, −80, −20, +10 mV, and +40 mV are shown as cyan, green, magenta, red, and blue, respectively. As theoretical traces are often directly superimposed on experimental data, we use gray boxes to indicate ranges of fitted experimental data.
with sequential binding of Na ions readily fit the experimental data. In comparison, a connectivity (5, e) selected to further investigations.

connectivity, models incorporating cooperative binding were which is, in fact, equivalent to the simultaneous binding of 2 orders of magnitude faster than the binding of the first Na binding is allowed, the best parameter sets found by the algorithm involved a very fast kinetic rate associated with the binding of the second Na ion (consistently between 1 and 2 orders of magnitude faster than the binding of the first Na), which is, in fact, equivalent to the simultaneous binding of 2 Na ions. As we want to generate the simplest possible connectivity, models incorporating cooperative binding were selected to further investigations.

Five 7-state models were then generated. Two of them (Fig. 5, e and f) still incorporated sequential binding of Na ions, and confirmed the previous findings (i.e., sequential binding is acceptable if the second Na ion binds at least 10 times faster than the first one). The remaining three cooperative models (Fig. 5, g–i) readily yielded good fit, each with various clusters of kinetic parameters sets. We define clusters of kinetic parameters sets as groups of kinetic parameters that are near each other in the kinetic parameters space. The fact that a good fit can be reached with several different clusters of kinetic parameters suggests that the connectivity is still overdefined.

Thus, four 6-state models were generated (see Fig. 5, j–m), each incorporating different basic characteristics of the three successful models of the previous generation (Fig. 5, g–i). They each generated good results in the sense that they could yield clusters of kinetic parameters that could reproduce most of the experimental data but not all. This suggests that the connectivity has become oversimplified.

Up to now, all connectivities were tested with symmetrical energy barriers (all αi = 0.5). Relaxing this constraint produces variable effects on the fitting procedure. For 7- and 8-state models, resulting parameter sets where αi was allowed to vary between 0 and 1 were examined but rapidly discarded. This is because usage of adjustable αi provided additional degrees of freedom which cause those kinetic models to fail to converge toward a consistent parameter set. Thus, only kinetic models with αi = 0.5 were considered for these models. As 6-state models without the use of adjustable αi failed to properly reproduce the complete experimental data set, 6-state models with variable αi were considered and were found to yield interesting results. Usage of asymmetric energy barriers with 6-state models yielded good reproduction of the experimental data set using connectivities j and m, and very good reproduction using connectivity k.

Once again, a set of new models with one fewer state was generated keeping the possibility of varying αi. Four 5-state connectivities were generated. Of those, connectivity p consistently yielded the most accurate results. Finally, 4 and 3-state connectivities were tested but both failed to adequately reproduce experimental data. Thus, connectivity p (see Fig. 5 and 6A) was identified as the optimal connectivity, i.e., the simplest model that is capable of reproducing the complete experimental data set.

**Optimal kinetic model.** The optimal kinetic model (connectivity p) is shown in Fig. 6A. This 5-state model is defined by 10 rate constants, 5 fractional charges (zi), 5 αi, and the total number of transporters per oocyte (N). This is a total of 21 parameters. To be able to visualize and compare clusters of parameters, it is useful to regroup some of these into “metaparameters”. Metaparameters should reflect significant general characteristics of a model and allow a reliable identification of similar models. We choose to plot, in a three-dimensional graph, the final value of the cost function as a function of Σabs(zi) and N. While the sum of all transferred charge is always close to −2 because 2 Na ions are transported per MI, Σabs(zi) may be larger than 2 whenever some voltage-sensitive steps have positive z, i.e., a step for which a negative membrane potential would tend to reduce the turnover rate. The number of transporters per oocyte is also a very important parameter as it is inversely related to the turnover rate or to the speed of the rate-limiting step. As shown in Fig. 6B, the 32 simulations made with model p have yielded four distinct clusters of kinetic parameters using the chosen metaparameters.
representation. The blue cluster, which contains 12 sets of parameters out of 32, was selected as it has the lowest cost function and is likely to represent the global minimum. Costs of lumping two consecutive reactions (one of them being voltage independent, (9), but the voltage dependency of this parameter has not been previously investigated. Here we show that Pz efficiency is lost by 10.22.0.33.4 on November 8, 2016 http://ajpcell.physiology.org/ Downloaded from

DISCUSSION

Incomplete inhibition of pre-steady-state currents by Pz. Analyzing the kinetic mechanism of SMIT2 represents a good challenge as many difficulties have first to be dealt with to have a good set of pre-steady-state and steady-state current measurements.

To identify the currents coming exclusively from SMIT2, it is important to have a reliable specific inhibitor. The inhibitory constant of Pz for SMIT2 was reported as 76 μM at −50 mV (9), but the voltage dependency of this parameter has not been previously investigated. Here we show that Pz efficiency is lost after spending 300 ms at positive Vm. This prevents using Pz to identify SMIT2-specific transient currents in this voltage range. Fortunately, replacing external Na+ with NMDG seems to prevent all charge displacement associated with SMIT2 and brings the sigmoid Q-V curve of oocytes expressing SMIT2 down to a linear relationship as expected from a pure membrane capacitive current. The transient currents measured in the absence of Na+ were then used to subtract the capacitive currents from the transient currents generated by SMIT2.

Table 1. Kinetic parameters

<table>
<thead>
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<th>k_{forward}</th>
<th>k_{backward}</th>
<th>α</th>
<th>z</th>
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<tbody>
<tr>
<td>C1+C2</td>
<td>4.58 ± 0.03 s(^{-1})</td>
<td>13.83 ± 0.05 s(^{-1})</td>
<td>0.998 ± 0.001</td>
<td>-0.304 ± 0.003</td>
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<tr>
<td>C2+C3</td>
<td>220 ± 3 s(^{-1})</td>
<td>231 ± 4 s(^{-1})</td>
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<td>0.006 ± 0.006</td>
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<td>C3+C4</td>
<td>(2.29 ± 0.04) × 10^4 M/s</td>
<td>(141.9 ± 0.81) s(^{-1})</td>
<td>0.8990 ± 0.0007</td>
<td>-0.851 ± 0.004</td>
</tr>
<tr>
<td>C4+C5</td>
<td>(1.83 ± 0.02) × 10^4 M/s</td>
<td>(8.5 ± 0.19) s(^{-1})</td>
<td>0.989 ± 0.008</td>
<td>-0.195 ± 0.003</td>
</tr>
<tr>
<td>C5+C1</td>
<td>3.25 ± 0.03 s(^{-1})</td>
<td>(3.59 ± 0.04) × 10^4 M/s</td>
<td>0.996 ± 0.002</td>
<td>-0.656 ± 0.003</td>
</tr>
<tr>
<td>N</td>
<td>(1.464 ± 0.002) × 10^11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 12, based on the blue cluster presented in Fig. 6B. SE values should not be interpreted as directly correlated with the error on the parameters, but instead as a confirmation of the convergence of the fit. N, total no. of transporters/oocyte; C1–C5, the 5 states of model p in Fig. 6.
Concerning the steady-state cotransport currents, they can be measured and modeled as the MI-dependent currents without having to rely on Pz.

**SMIT2 generates a Na-sensitive leak current.** For a cotransporter, a leak current is defined as a transporter-specific current observed in the absence of substrate. The presence of leak currents has been identified for members of the SLC5 gene family (6, 8, 9, 11) as well as for other members of the LeuT structural family [LeuT (37) and DAT (20)]. We recently revisited the leak of SGLT1 (25) and found that it can be mediated by a variety of monovalent cations including H\(^+\), Na\(^+\), Li\(^+\), K\(^+\), and Cs\(^+\). This indicates that the cations involved do not have to interact with the Na\(^+\)-binding site to participate in the leak current. Following the crystallization of the bacterial homolog vSGLT (13), a water-filled pathway composed of ~100 water molecules was identified in both the vSGLT crystal structure and a hSGLT1 homology model (35). The presence of passive water permeation has also been shown in members of the SLC5 gene family (SGLT1) and the SLC6 gene family (GAT-1 and LeuT) (34) and in EAAT1 (30). Taken together, these studies support the contention that the leak current is mediated by the flow of cations through a channel-like pathway filled with water and establishing an almost continuous communication between the extracellular and intracellular milieus. Even if the permeability of this pathway for water and cations is Pz-sensitive and may be modulated by the specific conformation of the cotransporter, it is important to state that, in this view of the leak current, the flux of ions is not controlled by the conformational changes and the binding steps that produce cotransport.

In the case of SMIT2, within the voltage range where Pz is effective (from −155 to −5 mV), the leak can be measured as a Pz-sensitive current in the absence of MI. In the presence of external Na\(^+\), the leak current is relatively large as it can reach ~50% of the maximal Na-MI cotransport current (compare Fig. 3A and Fig. 7C). This is in contrast with SGLT1 for which the leak current is typically <10% of the cotransport current (25). Surprisingly, when the external Na is progressively replaced by NMDG, the leak current measured at −155 mV further increases by up to fivefold. The inhibiting effect of external Na\(^+\) appears to be Michaelian with an apparent affinity of 10 mM (estimated at −155 mV) and a maximal effect that would bring the leak current down to 10% of its initial amplitude at 0 mM Na\(^+\). It is possible that this Na\(^+\) dependence comes from the fact that some of the Na-bound conformations of the cotransporter are less “leaky” than the Na-free conformations. On top of its large amplitude, the leak current measured at \(V_m\) more negative than −110 mV is clearly time dependent (see Fig. 2) and contaminates the measurement of the true cotransporter pre-steady-state current. This is why we chose to eliminate from our data set the currents recorded at a \(V_m\) more negative than −110 mV (as shown in Fig. 4). Fortunately, the OFF currents are much less contaminated by this time-dependent leak current as the amplitude of the leak current in minimal at −50 mV. This is why we kept in the data set the full voltage range for the \(Q_{off}\)-\(V_m\) curves (as shown in Fig. 7, A and B).

**Analysis of the kinetic models obtained.** Using a systematic approach to determine the optimal connectivity (see Fig. 5), we were able to identify the simplest model capable of closely reproducing the experimental data as shown in Figs. 4 and 7. A number of conclusions can be drawn from this result, but also from the strategy that has led to it.

First, it was possible to properly describe steady-state and pre-steady-state currents, for an extended voltage range and in the presence of various Na\(^+\) and MI concentrations. In comparison with previous models proposed for SGLT1, a larger set of experimental conditions was covered (7, 15, 26–28, 32), with fewer states (15, 26–28, 32), and with a more accurate reproduction of the actual experimental currents (7, 15, 27, 28, 32). This supports the idea that directly fitting the currents through SA while systematically comparing multiple connectivities is a valuable approach.

Careful examination of the rejected connectivities is also informative. From rejected 8-state models \(a\) and \(d\), and 7-state models \(e\) and \(f\), we show that the cooperative binding is a sound description for the binding of Na\(^+\) ions, at least within the millisecond timescale considered (down to 3–5 ms). This is also consistent with a reported Hill coefficient of 1.4 for Na\(^+\) binding to SMIT2 (9).

From rejected 5-state models \(o\) and \(q\), we show that at least three transitions are needed between the binding of MI of one
transport cycle and the binding of the Na ions of the subsequent cycle. This is also consistent with recent models which found that using five (26), six (27), and three (15) transitions was necessary to properly model the translocation of the substrate to the intracellular side, unbinding of the substrate, and exposure of the binding site to the extracellular side.

Although the states identified in a kinetic model do not have to match the states that could be resolved in X-ray crystallography, it is interesting to compare a kinetic model that reproduces the activity of a cotransporter with the structural mechanism emerging from the several recently available crystal structures. On the basis of structural studies of several members of the LeuT structural family, the alternate-access mechanism would work with a series of nine different states (labeled $S_1$ to $S_9$) as illustrated in Fig. 6A. The binding sites are first accessible from the extracellular side only (state $S_1$). Na$^+$ ions bind first to the cotransporter, producing a state (transition $S_1 \rightarrow S_2$) in which sugar is allowed to bind. Binding of MI (transitions $S_2 \rightarrow S_3$) induces closing of the extracellular gates (transitions $S_3 \rightarrow S_4$), leading the major cotransporter reorientation (closing of the extracellular vestibule and opening of the intracellular vestibule, transition $S_4 \rightarrow S_5$). This is followed by the opening of the intracellular gate, allowing intracellular release of substrate ($S_5 \rightarrow S_6$). The empty transporter is then believed to experience similar transitions involving closing the intracellular gate (transition $S_7 \rightarrow S_8$), a major conformational change (transition $S_8 \rightarrow S_9$), and opening of the extracellular gate (transition $S_9 \rightarrow S_1$), which bring the cotransporter back to its initial configuration (36). On the basis of the Na$^+$ and MI binding steps, states $C_2$, $C_4$, and $C_5$ of model $p$ have been aligned with the states $S_1$, $S_2$, and $S_3$ of the alternate access mechanism. As the two slowest reactions rates are from $C_1$ to $C_2$ and from $C_5$ to $C_1$, it is likely that $C_1 \rightarrow C_2$ and $C_5 \rightarrow C_1$ are related to major conformational changes: $C_1$ presenting an inward-facing vestibule while $C_2$ and $C_3$ would have an outward-facing vestibule. According to this scheme, the transition from $C_2$ to $C_3$, which was found to be the only voltage-independent transition, could be associated with the opening of the extracellular gate.

**Kinetic parameters.** One of the most striking characteristics of the proposed kinetic model is the strong asymmetry of the voltage dependency of each electrogenic transition. Structurally, this would require a charged and mobile residue (or an ion) to see a strong electrical field in an initial conformational state and a much weaker field in a final conformational state. Various processes can be devised to obtain this result, such as aqueous crevices (4), helical screws (17), or shielding by specific residues or whole protein domain. Nevertheless, while we cannot disprove the occurrence of these processes, it is possible to explain the strong asymmetry in voltage dependency on purely kinetic grounds. Representing a complex cotransport mechanism with a minimal number of states clearly requires lumping several contiguous states ($C_i$, $C'_i$, $C''_i$, etc.) into a single state ($C_i$) or ignoring the presence of a weakly populated state $C^*$ between $C_1$ and $C_{i+1}$. In this case, a slow kinetic transition between $C_i$ and $C^*$ could mask the presence of a subsequent fast transition between $C^*$ and $C_{i+1}$ (22). If the transition between $C_i$ and $C^*$ is voltage dependent while the transition between $C^*$ and $C_{i+1}$ is not, neglecting the presence of $C^*$ could lead in certain cases to a voltage-dependent transition from $C_i$ to $C_{i+1}$ and a voltage-independent transition from $C_{i+1}$ to $C_i$.

In the case of SMIT2, the large asymmetry of the electrogenic reaction rates indicates that it is possible to accelerate the cotransport turnover rate by applying a more negative $V_m$. This is clearly observed experimentally (Fig. 4, F–J). On the other hand, applying a more positive $V_m$ could not produce any strong outward cotransport current as none of the electrogenic transitions is sensitive to $V_m$. This is clearly supported by experimental observations in our laboratory where no outward cotransport current could be detected even after loading the oocytes with MI (through transport or injection) in the presence of a normal or of an increased intracellular Na$^+$ concentration after overnight Na-K-ATPase inhibition by K-free solution (unpublished observations). In the case of SMIT2, the cotransport current is so strongly inward rectifying that a reversal potential could never be accurately measured in all the conditions tested. This is why the transport stoichiometry of SMIT2 (3) could not be determined by detecting the cotransport reversal potential as it could be easily done with SGLT1 (5).

Interestingly, model $p$ with the rate constants given in Table 1 is consistent with this inward-rectifying characteristic of SMIT2. As shown in Fig. 7C, increasing intracellular concentrations of MI and Na to 0.5 and 30 mM, respectively, produces a relatively weak outward current in the observable positive voltage range. This is mainly due to the fact that the rate constant $k_{15}$ is rate limiting and voltage independent.

Another interesting result is the identification of the steps through which the cotransporter can harvest the electrical energy contained in $V_m$. During one forward turnover of the cotransporter, two positive charges will cross the entire membrane electrical field releasing an energy of $-10 \text{ kJ/mol} \times 50 \text{ mV}$. This can be either exclusively carried by the two Na$^+$ ions or the responsibility can be shared between several reactions where a charged fragment of the cotransporter can move in the membrane electrical field. The latter is clearly the case for SMIT2. The transitions of the empty carrier from $C_1$ to $C_3$ accounts for $\sim 15\%$ of the overall charge displacement (see $z_i$ values in Table 1). The Na$^+$-binding reaction accounts for $42\%$ of the charge displacement, which leaves 43% for the conformational change of the fully loaded carrier and intracellular Na$^+$ and MI release. For SGLT1, the corresponding proportions were 55% (empty carrier), 10% (Na$^+$ binding), and 35% (fully loaded carrier and intracellular release)(26). Even if the relative importance of the conformational change of the empty carrier appears to change between SMIT2 and SGLT1, both models agree on a significant contribution of the fully loaded carrier to the total charge displacement. This is in sharp contrast with the previously used assumption of an empty carrier bearing two mobile negative charges which would be totally neutralized after the binding of two Na$^+$ ions. This simplifying assumption was originally made in the first kinetic model proposed after the cloning of SGLT1 (32) and was kept in several subsequent papers dealing with SGLTs kinetic modeling (12, 19, 27, 28).

The forward rate-limiting step of the proposed model is the $C_1 \rightarrow C_2$ transition with a rate constant of 4.6 s$^{-1}$ at $V_m = 0 \text{ mV}$. At $-155 \text{ mV}$, the calculated turnover rate with saturating substrate concentrations is $\approx 21 \text{ s}^{-1}$ ($k_{12} \approx 29 \text{ s}^{-1}$ and $k_{51} \approx 182 \text{ s}^{-1}$ at this very negative $V_m$). At $-50 \text{ mV}$, the other slow forward transition of the model is $C_5 \rightarrow C_1$ (with $k_{51} \approx 12 \text{ s}^{-1}$).
This is consistent with the possibility that the major conformational step (a reorientation from outward to inward facing) is included in this transition (see Fig. 6A).

The transport mechanism of SMIT2 vs. its physiological role. The role of SMIT2 is to mediate MI absorption in the small intestine and its reabsorption in the proximal tubule (24). The normal MI concentration in the blood is around 50 μM and the filtered load is almost completely reabsorbed by the proximal tubule and the short loop of Henle (40). We have previously shown that SMIT2 uses a 2 Na⁺-1 MI stoichiometry to perform the difficult task of basically reabsorbing all MI from the tubular fluid (3). The features of the kinetic model that we propose are fully consistent with this physiological function, and it gives further details on the mechanism used by SMIT2 to exploit the energy present in the electrochemical gradient of Na⁺ to activate MI transport. In the forward direction, the cotransporter was shown to experience two extremely slow conformational changes (from C1 to C2 and from C3 to C1) which are likely to correspond to the major conformational changes that are required to alternatively expose the binding sites to the extracellular or to the intracellular side of the membrane. In the absence of external MI, the time course of the observed transient currents indicates that the conformational change from inward facing to outward facing is performed in at least two steps (within the typical 2-ms time resolution of the experiments), the first one (C1 to C2) being associated with a significant charge displacement. This is one of the steps where the energy of the membrane potential is used to activate the cotransporter. The binding of two extracellular Na⁺ ions can be described as a single step (C3 to C4) which is required to allow MI binding (C4 to C5). This is consistent with a highly cooperative binding step where the binding of the first Na favors the binding of the second ion in such a way that they appear to bind simultaneously. This binding step is associated with the largest charge displacement observed in the whole kinetic cycle. This could mean that the Na ions feel an ion-well effect as a fraction of the membrane electrical field is seen when Na ions move toward their binding sites. Alternatively, it can represent a voltage-sensitive conformational change that is triggered by the binding of the Na ions. This later possibility is probably the case for explaining the slight voltage dependence of the MI-binding step. In our experience, the intracellular Na⁺ and MI concentrations are very low and the cotransport process can be considered as occurring in zero-trans conditions. In this condition, the slow conformational change from outward facing to inward facing will dissipate all, possibly faster, steps involved in the intracellular release of Na⁺ and MI. This last step from C2 to C1 was observed to be voltage dependent, indicating that some of the lumped steps in this complex rearrangement are (is) carrying a charge displacement. All these voltage-dependent steps are organized in such a way that a negative membrane potential will accelerate the forward steps, leading to the cellular uptake of one MI and two Na⁺ ions. This could be rationalized as follows: the empty cotransporter carries a mobile negative charge that moves outside during the conformational change from C1 to C2. The binding of two Na ions is associated with the displacement of positive charges toward the intracellular side of the membrane. The negative mobile charge of the free carrier becomes a positive charge after Na⁺ binding, and this charge moves inward upon MI binding and during the major reorientation of the cotransporter (step C2 to C1).

Conclusion. In this study, we used the two-electrode voltage-clamp technique to measure the steady-state and pre-steady-state currents of SMIT2. Phlorizin was shown to be a poor inhibitor of SMIT2 at positive membrane potentials; however, replacing extracellular Na⁺ by NMDG was found to be efficient in blocking the transient currents generated by SMIT2. SMIT2 was also shown to have an exceptionally large leak current that is inhibited by extracellular Na⁺. After very negative $V_m$ pulses, this leak current is decaying with time and this creates a threefold difference between apparent $Q_m$ and $Q_{off}$ measured as the area under the transient current traces. Using a novel approach to kinetic modeling based on a simulated annealing algorithm, we proposed a 5-state model that accounts for SMIT2 electrogenic properties for various substrate concentrations and $V_m$. This model includes cooperative binding of Na⁺ ions, strong apparent asymmetry of the energy barriers, a rate-limiting step that is believed to be associated with the translocation of the empty transporter, and a low turnover rate of 21 s⁻¹ at very negative $V_m$. This study demonstrates the efficiency of the simulated annealing algorithm in testing different connectivities and in resolving the values of the numerous parameters involved in this detailed kinetic models.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

L.J.S. and J.-P.L. conception and design of research; L.J.S. and B.W. performed experiments; L.J.S. analyzed data; L.J.S. interpreted results of experiments; L.J.S. prepared figures; L.J.S. drafted manuscript; L.J.S. and J.-Y.L. edited and revised manuscript; L.J.S. and J.-Y.L. approved final version of manuscript.

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