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Rate control within the Na^+/glucose cotransporter

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Abstract

The intestinal Na^+/glucose cotransporter has recently been cloned and expressed in *Xenopus* oocytes, and kinetically characterised to produce a 6-state model of the transporter. We have analyzed this model using control analysis to determine the extent to which each rate constant and step in the mechanism limits the steady-state rates under various conditions. We show that control (rate limitation) is distributed among a number of rate constants and changes with the membrane potential and the concentrations of external and internal sodium and sugar. There is no single rate-limiting step but, with saturating concentrations of external sugar and sodium and negligible internal concentrations, the sugar and sodium fluxes are limited by sodium dissociation on the inside, translocation of the unloaded carrier to the outside and translocation of the fully loaded carrier to the inside. With more physiological external and internal concentrations there is a significant leak flux, which causes the cotransporter to act as a sugar uniporter, and entails that control over the sugar flux differs from control over the sodium flux. In these conditions control is widely distributed among many of the rate constants. The analysis shows that the concept of a rate-limiting step is not generally applicable to transporters.

Keywords: Metabolic control analysis; Rate-limiting step; Glucose transport; Sodium transport

1. Introduction

One of the main aims of the kinetic analysis of enzyme and transport proteins is the identification of steps or factors within the protein which limit the steady-state rate. This is important for understanding the control, regulation, evolution and function of the protein, and potentially for the genetic engineering of the protein. The analysis of rate limitation within enzymes and transporters has in the past been directed towards the identification of a rate-limiting or rate-determining step, and has used methods designed to identify such a step [1–3]. However such an analysis makes the assumption that an enzyme or transporter will always have a single, unique, and fully rate-limiting step under all conditions, or at least under any particular condition. Recent work [4] has shown that this assumption does not hold true for all soluble enzymes. Several steps within an enzyme may be partially rate limiting, and the distribution of control (rate limitation) between these steps changes in many conditions. Thus we have advocated the quantitative analysis of the extent to which different steps or rate constants limit the steady-state rates of enzymes and transporters [4]. We and others have

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provided a theoretical framework for such an analysis [4–6] derived from Metabolic Control Analysis. This analysis provides both a practical means of quantifying the extent of rate limitation and a way of relating this rate limitation to other enzyme parameters [7,8].

Na^+/glucose cotransporters are located in the epithelia of the small intestine and kidney cortex, and are responsible for the absorption of glucose and other sugars into the body and excretion of glucose from the body, respectively. Control of these transporters is important not just for carbohydrate homeostasis but also for the hydration state of the body, as the sodium flux via these transporters is significant in terms of the osmotic balance of the body. The Na^+/glucose cotransporter of rabbit small intestine has been identified and the cDNA cloned and expressed in *Xenopus laevis* oocytes (reviewed in [9]). Recently the kinetics of this cloned cotransporter have been characterised in detail using electrophysiological techniques to measure steady-state and pre-steady-state currents as a function of external Na^+ and sugar (α-methyl-D-glucopyranoside, αMDG) concentrations and membrane potential [10]. These innovative studies have been used to produce a 6-state kinetic model of the cotransporter, which reproduces all the kinetic properties measured experimentally; and a unique numerical solution was found for the 14 kinetic constants which accounted quantitatively for the experimental observations [11]. This kinetic model was used to identify rate-limiting steps within the cotransporter. According to Parent et al. [11] the rate-limiting step for sugar transport in their model is a function of the membrane potential, and external sodium and sugar concentrations. And according to Wright [9] the model indicates that with physiological levels of these parameters the rate-limiting step is the recycling of the unloaded carrier across the membrane.

However these conclusions from the experiments and model were reached within the assumption or paradigm of a single rate-limiting step. And this rate-limiting step was probably identified by the usual procedure of determining the step within the model with the lowest forward rate constant. However this procedure is defective in identifying rate-limitation [4,5]. In this paper we have therefore used control analysis to determine the extent to which each rate constant and step within the model of Parent et al. [11] limits the steady-state rate of the cotransporter. Our aims are: (a) to elucidate rate limitation within the Na^+/glucose cotransporter, (b) to determine whether transporters have rate-limiting steps, and (c) to demonstrate the power of control analysis in analysing rate limitation.

2. Theoretical background

Enzyme and transport reactions can be analyzed in terms of subreactions or steps between intermediate states of the protein, and kinetically characterised in terms of rate constants for these transitions. The extent to which a particular rate constant (e.g. k_i) limits the steady-state rate (v) of the enzyme/transporter can be quantified as a control coefficient defined as:

$$C^v_{k_i} = \frac{\partial v}{\partial k_i} \cdot \frac{k_i}{v}$$

This is just the percentage change in the steady-state rate divided by the percentage change in the rate constant causing that change, extrapolated to infinitesimally small changes. This is closely approximated by the percentage change in rate given by a 1% change in the rate constant. In any one particular condition each kinetic constant has a unique control coefficient over the rate, and the sum of these coefficients for all rate constants is always equal to 1. A high control coefficient means the rate constant limits the steady-state rate. A control coefficient close to zero means the rate constant does not limit the rate. Forward rate constants have positive control coefficients, backward rate constants have negative control coefficients (i.e. increasing the rate constant decreases the steady-state rate).

The control coefficient not only gives a consistent and unambiguous definition of the extent of rate limitation, but also is simply related to other enzyme parameters such as the rate constants, unidirectional rates and the thermodynamic disequilibrium of steps [7,8]. If we have a kinetic model and rate constants of an enzyme/transporter we can calculate the control coefficients directly under any condition by differentiating the rate equation with respect to each

rate constant [4], as the control coefficient is the normalised partial differential of the rate with respect to the rate constant (see above).

The rate limitation of a step (i.e. the backward and forward rate constants of a single transition) as opposed to a single rate constant can also be quantified as a control coefficient of the step defined as the normalised differential of the steady-state rate with respect to the two rate constants changed in proportion. This is approximated by the percentage change in rate for a 1% change in the rate constants. This coefficient is equal to the sum of the coefficients of the forward and backward rate constants for the step, and is always positive and between 0 (no control) and 1 (completely rate limiting) for an unbranched enzyme mechanism.

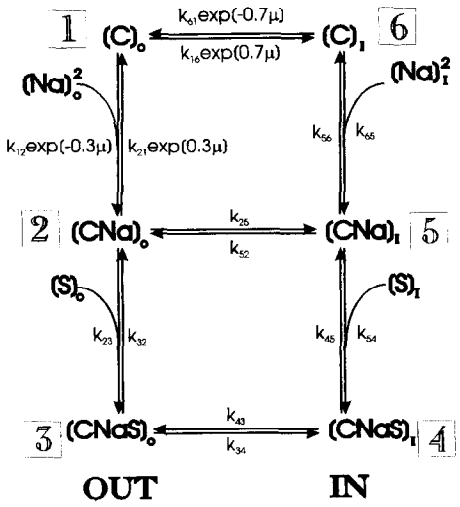

Fig. 1. Transport model devised by Parent et al. [11] for the Na^+/glucose cotransporter. The 6 states of the carrier are: (1) the unloaded carrier orientated to the outside of the cell $[C]_o$, (2) the Na^+ bound carrier orientated to the outside $[CNA]_o$, (3) the sugar and Na^+ bound carrier orientated to the outside $[CNaS]_o$, (4) the sugar and Na^+ bound carrier orientated to the inside of the cell $[CNaS]_i$, (5) Na^+ bound carrier orientated to the inside $[CNa]_i$ and (6) unloaded carrier orientated to the inside $[C]_i$. The number in boxes refer to the number of the state. These states are interconverted by 7 transitions, characterised by 14 rate constants labelled using the numbers and order of the states involved in the transition. The sugar transport occurs only in the presence of two sodium ions that bind first to the empty carrier. Transitions between states 1 and 2, and between states 1 and 6 are voltage dependent, and the rates are described by voltage-independent rate constants k_{12}, k_{21}, k_{16}, and k_{61} raised to the power of a voltage-dependent factor. μ is the electrochemical potential FV/RT. The constants 0.3 and 0.7 appearing in the exponentials are phenomenological constants that describe the fraction of the electrical field sensed by external sodium binding and by empty carrier translocation, respectively. The potential and ligand concentration independent rate constants are: k_{12} 80000 s^{-1} mol^{-2}, k_{21} 500 s^{-1}, k_{23} 10^5 s^{-1} mol^{-1}, k_{32} 20 s^{-1}, k_{34} 50 s^{-1}, k_{43} 50 s^{-1}, k_{45} 800 s^{-1}, k_{54} 4×10^7 s^{-1} mol^{-1}, k_{56} 10 s^{-1}, k_{65} 50 s^{-1} mol^{-2}, k_{61} 5 s^{-1}, k_{16} 35 s^{-1}, k_{25} 0.3 s^{-1} and k_{52} 0.3 s^{-1}. Adapted from [11].

The control coefficients quantify the extent to which the rate will change when one or more rate constants is changed. But control coefficients do not by themselves predict how the rate will change in response to some effector of the protein. This response also depends on the sensitivity ('elasticity') of the rate constants to the effector. The total response coefficient of the enzyme to the effector is the sum of the individual responses of the rates at each step, which in turn are the product of the control coefficient of each rate constant and its elasticity to the effector [4,7].

Some effectors (e.g. inhibitors and activators) can not change a single rate constant alone, because this would violate the principle of microscopic reversibility expressed in the Haldane relation [4,7]. This is not a constraint on the control coefficients but rather on the elasticities of an enzyme/transporter to effectors. However, when using control coefficients to predict rate changes and to understand rate limitation, it is important to consider how the change in rate constant (or rate constants) might be brought about and what other rate constants are likely to change. Otherwise the coefficients may be erroneously used to make unrealisable predictions. The control coefficients of rate constants should not be considered in isolation, but rather as elements within the response coefficient (see [4,7]).

3. The cotransporter model and its analysis

The kinetic model of the Na^+/glucose cotransporter produced by Parent et al. [11] consists of 6 states (see Fig. 1) — (1) the unloaded carrier orientated to the outside of the cell, (2) the Na^+ bound carrier orientated to the outside, (3) the sugar and Na^+ bound carrier orientated to the outside, (4) the sugar and Na^+ bound carrier orientated to the inside of the cell, (5) Na^+ bound carrier orientated to the

inside, and (6) unloaded carrier orientated to the inside. The rate constants were estimated with α-methyl-D-glucopyranoside (αMDG) as the transported sugar, and the number of sodium atoms transported per cycle was assumed to be 2. The 6 states operate sequentially in a single cycle to effect sugar and sodium cotransport. There is also a seventh transition between states (2) and (5) which effects a leak flux (or 'slippage' according to the bioenergetic terminology, see [15]). These 7 transitions are characterised by 14 rate constants estimated by Parent et al. [11] and given in the legend to Fig. 1. There are 3 steady-state rates for the model cotransporter of Fig. 1: the sugar flux (equal to the net rate between states 3 and 4), the leak flux (equal to the net rate between states 2 and 5), and the sodium flux (equal to the net rate between states 6 and 1). The sodium flux is always equal to the sum of the sugar flux and the leak flux. In many conditions the leak flux ('slippage') is negligible and thus the sugar flux equals the sodium flux.

We calculated the control coefficients of each rate constant of the cotransporter model over the steady-state rates of sugar and sodium transport for a range of concentrations of sodium and sugar and values of the membrane potential. We calculated the control coefficients by taking the rate equation and rate constants from Parent et al. [11], and differentiating the rate equation with respect to each rate constant, and multiplying the partial differential by the rate constant divided by the rate (as in the definition of the control coefficient given above). The steady-state rate of any one of the 3 fluxes is given by the forward unidirectional rate minus the backward unidirectional rate of any one step of a particular flux pathway. And the unidirectional rate of a step between two enzyme intermediate states is given by the concentration of the intermediate state multiplied by the rate constant. Expressions for the concentration of intermediate states in terms of all the rate constants are obtained by the King–Altman method as described in [11]. These expressions consist of a ratio of two sums of terms, and these terms are either zero order or first order with respect to any particular rate constant. Thus the two sums are easily differentiated with respect to these rate constants, and from this the differential of the ratio of the sums can be found. In practice this was done by writing a BASIC computer programme which calculated the sum in any particular condition, then subtracted from this the same expression evaluated with a particular rate constant set to zero (i.e. the zero order terms were subtracted off), and then the residue was divided by the rate constant to give the differential with respect to that rate constant. The differential of the rate equation for a particular flux could then be calculated.

A number of control coefficient values were also evaluated by SCAMP, a metabolic control analysis programme [12]. This programme takes the model and evaluates the control coefficients by numerical methods and thus gives entirely separate and independent evaluations of the coefficients. Where coefficients were evaluated by both methods they gave identical results.

4. Results

In Figs. 2–4 and 6 we have plotted the control coefficients of the individual rate constants or steps of the cotransporter model over the sodium or sugar flux calculated for particular values of the membrane potential and the external and internal sugar and sodium concentrations. In Fig. 2 the control coefficients over the sugar flux were calculated as a function of the membrane potential with saturating levels of external sugar and sodium, and negligible levels of internal sugar and sodium. Control over sodium flux and control over sugar flux are similar (but not identical) under the conditions of Fig. 2 because the leak flux (slippage) is relatively small. The figure shows that under these conditions control (rate limitation) is shared between a number of rate constants, and the distribution of control varies with the membrane potential in the range +50 to −150 mV. No one rate constant is uniquely rate limiting at any membrane potential over this range, but the dominant rate constants are those of unloaded carrier return (k_{61}) over the range +50 to −50 mV, and internal sodium dissociation (k_{56}) over the range −50 to −150 mV. This agrees qualitatively with the conclusions of Parent et al. [11] who stated that their model showed that k_{61} was rate limiting in these conditions at membrane potentials more positive than −43 mV and k_{56} was rate limiting at potentials more negative than −43 mV.

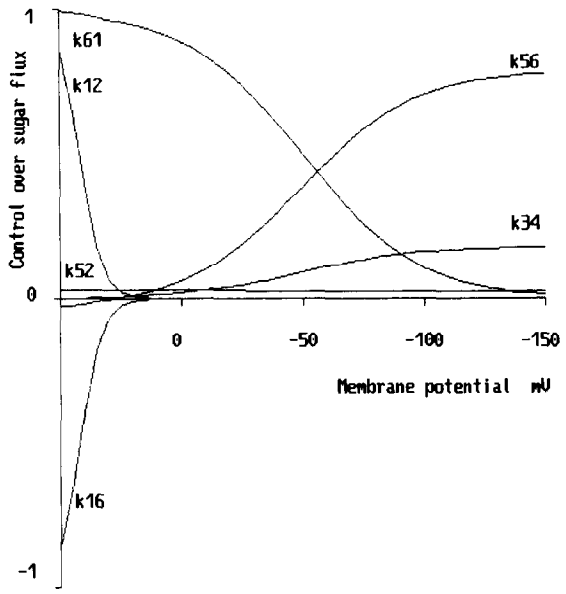

Fig. 2. Control coefficients of individual rate constants over sugar flux as a function of membrane potential with saturating concentrations of external sodium and sugar and negligible internal concentrations. The following concentrations were used to calculate the coefficients: external sodium = 100 M, external sugar = 1 M, internal sodium = 1 mM, and internal sugar = 1 mM. Internal concentrations were not set at zero as this would require division by zero in evaluating the coefficients. However, reducing the internal concentrations by orders of magnitude had no effect on the coefficients values, i.e. the internal concentrations were negligible.

However, even with 100 M external sodium the rate constant for external sodium binding (k_{12}) becomes limiting at positive membrane potentials, due to an effect of membrane potential on external sodium binding. With a more physiological level of external sodium (about 100 mM) k_{12} becomes partially rate limiting at potentials more positive than -100 mV, and k_{61} has less control. And at sodium concentrations below 10 mM k_{12} becomes almost exclusively rate limiting in these conditions. This agrees with the conclusions of Parent et al. [11]. Control over sugar flux and over sodium flux are similar in these conditions because the leak flux is relatively small.

Control by the enzyme steps is just given by the sum of the control coefficients of the backward and forward rate constants of that step. Thus in the conditions of Fig. 2 it is easily seen that the transition between states 3 and 4 (step 3/4) and step 5/6 have most of the control at the more negative membrane potentials, while step 5/6 has most of the control at potentials around zero, but looses control to step 1/2 at more positive potentials.

If the external sugar concentration is varied with saturating sodium concentrations and a membrane potential of -50 mV, the control coefficient of the rate constant of external sugar association (k_{23}) does not become significant until the sugar concentration falls below 1 mM, due to the high affinity of the cotransporter for sugar (not shown). The control coefficients of external sugar dissociation (k_{32}) and internal translocation of the fully loaded transporter (k_{34}) are also significant at low external sugar concentrations. However, if control over sodium flux rather than sugar flux is calculated in the same conditions, then as before the control coefficient of external sugar association starts to rise significantly below 1 mM external sugar, but below 10 μM it

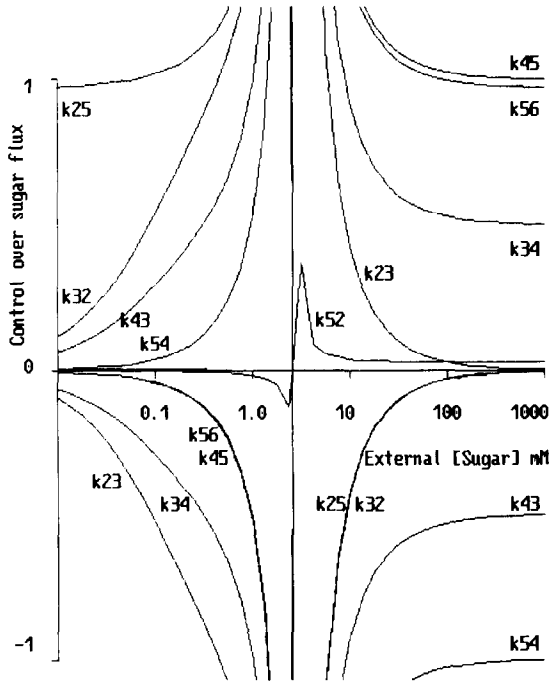

Fig. 3. Control coefficients of individual rate constants over sugar flux as a function of the external sugar concentration with 'physiological' concentrations of sodium and sugar. The following concentrations and potentials were used to calculate the coefficients: external sodium = 0.1 M, internal sodium = 10 mM, internal sugar = 10 mM, and membrane potential = 50 mV.

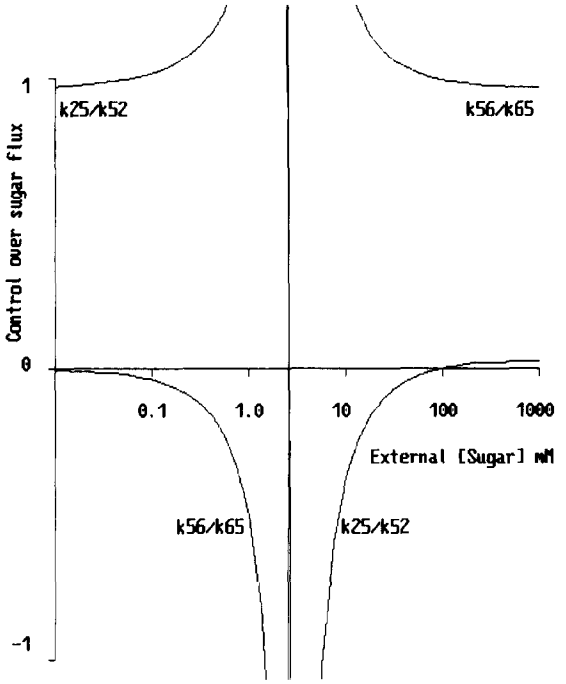

Fig. 4. Control coefficients of enzyme steps over sugar flux as a function of the external sugar concentration with 'physiological' concentrations of sodium and sugar. The following concentrations and potentials were used to calculate the coefficients: external sodium = 0.1 M, internal sodium = 10 mM, internal sugar = 10 mM, and membrane potential = −50 mV. The labels of the curves refer to the steps rather that the rate constants.

falls off again as the control coefficient of the leak (k_{25}) starts to rise. This is due to the leak flux becoming significant at limiting sugar concentrations.

In Fig. 3 the concentrations and potential are set at more 'physiological' levels that might be found for glucose in vivo, and are used to calculate the control coefficients over sugar flux. External sodium is set at 100 mM, internal sodium at 10 mM, internal sugar at 10 mM, membrane potential at −50 mV, and the control coefficients of individual rate constants are plotted as a function of external sugar concentration, which physiologically might be 10 mM or higher (for glucose) after a meal and lower between meals. The figure shows that control is widely distributed, and changes rather dramatically at external sugar concentrations between 0.1 and 100 mM. This is because the sugar flux changes direction over this range, due to the presence of a significant leak flux. In Fig. 4 we have plotted the control coefficients of the 7 transporter steps rather than the individual rate constants in exactly the same conditions as Fig. 6. We can see than rate limitation is confined to the internal sodium association/dissociation step and the leak step.

In Fig. 5 we have plotted the sugar flux, leak flux and sodium flux predicted by the model as a function of the external sodium concentration in the same ('physiological') conditions as Fig. 3. At high external sugar concentrations there is a coupled sugar influx and a very small uncoupled sugar influx (that is a sugar leak due to cycling between states 2, 3, 4 and 5). As the external sugar concentration is lowered the leak flux reverses, but there is still net sugar influx. However at still lower external sugar concentrations the transporter mediates uncoupled sugar efflux (via the cycling of states 2, 5, 4 and 3). Figs. 3 and 4 show that at possible physiological concentration of external sugar that might be encountered after a meal (between 1 and 50 mM) control over the sugar flux is shared between a large number of rate constants and is very sensitive to the external sugar

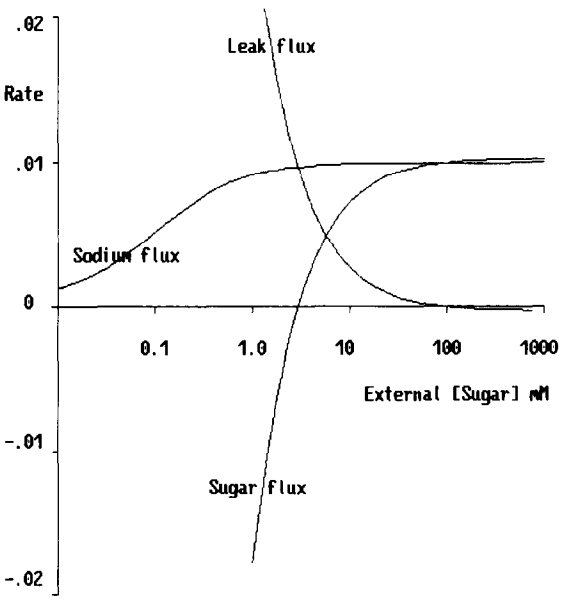

Fig. 5. The sugar, sodium and leak fluxes as a function of external sugar concentration with 'physiological' concentrations. The following concentrations and potentials were used to calculate the fluxes: external sodium = 0.1 M, internal sodium = 10 mM, internal sugar = 10 mM, and membrane potential = −50 mV.

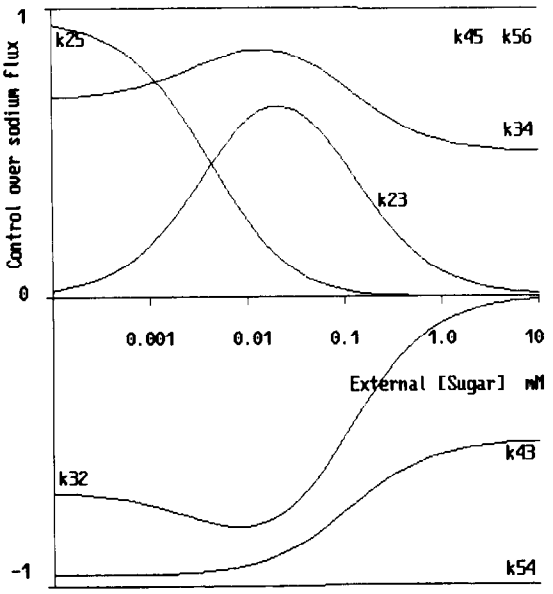

Fig. 6. Control coefficients of individual rate constants over sodium flux as a function of the external sugar concentration with 'physiological' concentrations of sodium and sugar. The following concentrations and potentials were used to calculate the coefficients: external sodium = 0.1 M, internal sodium = 10 mM, internal sugar = 10 mM, and membrane potential = − 50 mV.

concentration. The control coefficients of many of the rate constants and some of the steps approach positive and negative infinity as the sugar flux approaches zero. This is because a finite fractional change in these rate constants can cause an infinite fractional change in the rate when the rate is zero.

In Fig. 6 we have plotted the control coefficients of individual rate constants over the sodium flux (rather than sugar flux) in the 'physiological' conditions of Fig. 3. We can see that control over the sodium flux does not change so dramatically as for the sugar flux (Fig. 3). This is due to the fact that the sodium flux does not change greatly as the external sugar concentration is varied (Fig. 5).

5. Discussion

These findings for physiological type conditions differ from the conclusions of Wright [9]. Wright [9] concluded that "at physiological voltages and concentrations the rate-limiting step is the recycling of the unloaded carrier" (i.e. k_{61}). In fact we find that k_{61} has no significant control at anything approaching 'physiological' voltages and concentrations. It is unclear what a physiological concentration of sugar is, since the model rate constants were estimated from experiments with αMDG which is not a physiological sugar. However it has been determined in the same experimental system that the V_{max} and external K_m are similar for glucose, galactose and αMDG [9,13,14]. Thus we conclude that the distribution of control in physiological conditions as a function of the concentration of external D-glucose should look similar to Figs. 3 and 6. However we can not be certain that some of the rate constants with D-glucose are not radically different from those with αMDG, leading to a different control distribution.

Parent et al. [11] stated that the leak flux ('slippage') was insignificant in their model of the cotransporter. However they examined the predictions of their model principally in conditions of saturating concentrations of external sodium and sugar and zero internal concentrations of sodium and sugar. As we can see from Figs. 4 and 5 with saturating external sugar concentrations the leak flux (slippage) is indeed insignificant, but at lower external sugar concentrations (and perhaps more physiological conditions) the leak (slip) becomes very important for the control distribution, the rate and the direction of fluxes. The leak allows the cotransporter to also act as a sodium or sugar uniporter in particular conditions, and this might be significant for its physiological function. In fact the leak makes the sugar flux of the transporter much more sensitive to changes in rate constants and to the external sugar concentration over the possible physiological range than it would be in the absence of the leak. This might be a functional advantage for regulation of the transporter, as has been argued for leaks and slips in other transport proteins (see [15] and references therein).

In the review of Wright [9] the rate constants of the cotransporter are reported as being slightly different from those reported in Parent et al. [11] and this presumably reflects more recent work by this group on the kinetics of the cotransporter [16,17]. The effect of these slight adjustments of the rate constants is to make the leak flux even more significant.

Because sodium dissociation on the inside (k_{56}) has a high control coefficient over the fluxes in many conditions while sodium association (k_{65}) has no control, it is possible for an effector or other change to alter the fluxes by affecting this step alone. Thus this step might be an important physiological target for regulation by effectors internal to the cell. Physiological regulators of the transporter are not known at present, but it is suspected that the transporter may be phosphorylated on the inner side [9,18], and this could potentially affect sodium binding.

The fact that a rate constant has significant control over the steady-state rate of an isolated transporter or enzyme does not mean that step has significant control in vivo. This is partly because the substrate and product concentrations, which are fixed parameters in vitro, are variables in vivo determined by the kinetics of all the enzymes/transporters producing or consuming them. Thus the control coefficient of the whole Na^+/glucose cotransporter over the glucose and sodium fluxes via the transporter in vivo is not necessarily 1. Rather it will depend on the relative sensitivities (elasticities) of the cotransporter and other transporters of glucose and sodium to changes in the intracellular level of glucose and sodium [19]. The control coefficient of a rate constant over a pathway flux is equal to the control coefficient of the rate constant over the isolated enzyme rate multiplied by the control coefficient of the whole enzyme over the pathway rate in the same conditions [4].

The control analysis of the Na^+/glucose cotransporter has shown that control over the fluxes (rate limitation) is distributed among a number of particular rate constants, and that distribution of control changes in different conditions. In any one particular condition there may be one rate constant that has most of the control, but only in relatively rare conditions is all the control exclusively located in a single rate constant or step. Similar conclusions were reached from the control analysis of a number of soluble enzymes [4], and from more conventional methods of analysis of the Ca^{2+}-ATPase [2] and of a number of β-lactamases [20]. Thus the general assumption that a transporter or enzyme will have a rate-limiting or rate-determining step is incorrect, and efforts to identify such a step are misconceived. Control analysis provides an powerful alternative means of analysing rate limitation within proteins which does not assume the presence or absence of a rate-limiting step [4–8].

We have analyzed the cotransporter only in terms of limitation of the steady-state rates by the rate constants. Control analysis also provides a framework for analysing the control over the sensitivity to effectors, K_m, K_m/V_{max}, and the levels of intermediate states of the enzyme/transporter, by levels of substrate, product, membrane potential and other effectors, as well as by the rate constants and steps [4].

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