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Molecular analysis of the mechanism of potassium uptake through the TRK1 transporter of *Saccharomyces cerevisiae*

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Abstract

The TRK-HKT family of K^+ transporters mediates K^+ and Na^+ uptake in fungi and plants. In this study, we have investigated the molecular mechanism involved in the movement of alkali cations through the TRK1 transporter of *Saccharomyces cerevisiae*. The model that best explains the activity of ScTRK1 is a cotransport of two K^+ or Rb^+, both of which bind the two binding sites of ScTRK1 with very high affinities in K^+-starved cells. Na^+ can be transported in the same way but it exhibits a much lower affinity for the second binding site. Therefore, only at critical concentration ratios between K^+ and Na^+, or Rb^+ and Na^+, the transporter takes up Na^+ together with K^+ or Rb^+. Mutation analyses suggest that the two binding sites are located in the P fragment of the first MPM motif of the transporter, and that Gln^{90} is involved in these binding sites. ScTRK1 can be in two states, medium or high affinity, and we have found that Leu^{949} is involved in the oscillation of the transporter between these two states. ScTRK1 mediates active K^+ uptake. This is not Na^+-coupled and direct coupling of ScTRK1 to a source of chemical energy seems more probable than K^+–H^+ cotransport.

Keywords: Potassium transporter; Yeast; HKT1; TrkB

1. Introduction

Potassium is a major component of all living cells. Its concentration in the cytoplasm is approximately 10^{-1} M, 3–5 orders of magnitude higher than in soils, where soil fungi and plants roots compete for nutrients. This asymmetric distribution of K^+ across the plasma membrane has been a constant during the evolution of plants and fungi, which colonized the emerged lands 500 millions years ago.

The K^+ transporters of fungi and plants are of two types, HAK and TRK. Transporters of the HAK type seem to be present in all plants, but not in all fungi [1]. In contrast, TRK type transporters, which are called HKT in plants, are present in all the fungi and plants studied up to now [1] and are also present in most bacteria and archea [2]. In *Saccharomyces cerevisiae*, K^+ uptake is mediated by two TRK transporters, TRK1 and TRK2 [3], although the contribution of TRK2 is insignificant [4]. If the *TRK1* gene is disrupted, the mutant shows a defective K^+ uptake and is hyperpolarized; it exhibits a low rate transport mediated by TRK2 [4] and an ectopic K^+ uptake of low affinity mediated by many different transporters [5]. If both *TRK1* and *TRK2* are disrupted, the double mutant exhibits exclusively the ectopic, low-affinity K^+ uptake [4].

In the absence of a systematic study of eukaryotic TRK-HKT transporters, there are several questions regarding the structure and function of these transporters that are far from being solved. With reference to the structure, sequence analyses support the notion that the protein structure of the TRK-HKT transporters contains four repeated motives [2], and resembles the structure of the KcsA channel of *Streptomyces lividans*, which is made up of four identical subunits. The KcsA protein has two transmembrane α-helices connected by the roughly 30-amino-acid P segment, in which the selectivity filter of the channel is located [6]. According to this, the TRK-HKT transporters have a tetra-M1PM2 structure, instead of a 12-transmembrane fragment structure, as hydrophobicity studies suggest.

In terms of function, it seems that the TRK type transporters mediate the symport of two ions, typically K^+ and Na^+, although they evolved by gene duplication and fusion of molecular structures that were originally K^+ channels [2,7]. The TaHKT1 wheat transporter fits into this model, because it cotransports K^+ and Na^+, but the AtHKT1 transporter seems to transport only Na^+, and all fungal

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TRK transporters seem to transport only K^+. In particular, K^+ uptake in *S. cerevisiae*, which is mediated by the ScTRK1 transporter, has been extensively studied for many years, and there is no report suggesting that it can be a K^+–Na^+ symporter [1,8]. Interestingly, the concentration dependence of K^+ or Rb^+ influxes in *S. cerevisiae* deviate from a Michaelis–Menten equation at low cation concentrations. This response is not found in uniporters with a single binding site, but can be explained if the transporter has two binding sites, and it has proposed that one of them is an activation site [8]. However, the kinetics could be also explained if both sites participate in transport, and ScTRK1 functioned as a peculiar uniporter which cotransported two identical ions per cycle [1].

In view of the questions posed by the scarcity of mechanistic data in eukaryotic TRK-HKT transporters, we have initiated a systematic study to determine the structure and function of the ScTRK1 transporter. We selected the *S. cerevisiae* transporter because the best tool to study these transporters is a *S. cerevisiae trk1 trk2* mutant, and the study of ScTRK1 avoids the possible problems that heterologous expressions may produce.

We here report kinetic analyses, physical data and mutant analyses which support that ScTRK1 has two cation transport sites and that it normally cotransports two identical ions.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The *Escherichia coli* strain DH5α was routinely used for plasmidic DNA propagation. The yeast strain WΔ3 (*Mat***a** *ade2 ura3 trp1 trk1Δ::LEU2 trk2Δ::HIS3*) [9], deficient in the endogenous K^+ uptake systems, was used for expressing the transport systems assayed in this report. The ScTRK1 transporter was expressed from plasmid pRH22 [4], and the AtAKT1 channel from plamisd pFL61 [10]. Yeast transformants were grown in SD medium [11] supplemented with 25 mM K^+, but growth experiments were carried out in arginine phosphate (AP) medium [12] supplemented with the indicated K^+ concentrations. Wild-type K^+-starved cells were obtained by transferring actively growing cells in 3 mM K^+ AP-medium, into K^+-free AP medium, and incubating them for 5 h. Azide treated cells were obtained by exposing actively growing cells to 10 mM Na^+ azide in K^+-free AP medium for 10 min [13]. In the case of mutants, we followed similar protocols, except that they were grown at 15 mM K^+ AP medium.

2.2. DNA manipulations and mutant isolations

Manipulation of nucleic acids was performed by standard protocols or, when appropriate, according to the manufacturer's instructions. Random mutations in the *ScTRK1* gene were introduced by transforming plasmid pRH22 into a mutator *E. coli* strain (Epicurian coli XL1-Red, Stratagene), which is deficient in three of the primary DNA repair pathways, and then following the instructions of the manufacturer. The resulting plasmids were transformed into the WΔ3 strain, and mutant clones were selected by screening the yeast transformants at 0.2 or at 0.5 mM K^+ plus increasing Na^+ concentrations up to 350 mM in AP medium. The Q90R mutant here described did not grow at low K^+ and was Na^+-sensitive; the L949P mutant was Na^+-sensitive but grew at low K^+. The plasmids in the selected clones were isolated, and the inserts sequenced by using an automated ABI PRISM 377 DNA sequencer (Applied Biosystems).

2.3. Transport assays

For K^+ uptake experiments, K^+-starved cells were suspended in testing buffers: 2% glucose, 10 mM MES, for pH 6.0, HEPES, for pH 7.2, or TAPS, for pH 8.0 brought to pH with solid $Ca(OH)_2$. K^+ was added to the buffer, and the depletion of K^+ was followed in the external medium by atomic emission spectrophotometry, after a rapid centrifugation of the samples. For regular Rb^+ and Na^+ uptake experiments, cells were suspended in testing buffers and, at intervals after addition of Rb^+ or Na^+, samples were taken, filtered through 0.8-μm pore nitrocellulose membrane filters (Millipore) and washed with 20 mM $MgCl_2$ in the same filter. This washing eliminated the nonspecifically bound cations. Filters were incubated overnight in 0.1 M HCl, and Rb^+ or Na^+ was determined by atomic emission spectrophotometry of acid extracted cells. The initial rates of Rb^+ or Na^+ uptake were determined from the time courses of the cellular Rb^+ or Na^+ contents. In experiments at low Na^+ and Rb^+ concentrations (<50 μM), the procedure of cation depletion described for K^+ was followed.

2.4. Analysis of the data

All the experiments were normally repeated four times. The agreement among repetitions was high and typically the standard deviations were lower than 10% of the mean. The data in Tables 1, 4 and 5) are means of independent repetitions. Each condition in Fig. 1 corresponds to a single experiment, but repetitions produced results almost identical to those presented. In Fig. 3, each datum point corresponds to the result of a single experiment, but the whole range of concentrations was constructed with the results of two or three independent experiments because, for technical reasons, the same batch of cells cannot be used to generate the data corresponding to the complete range of concentrations. This applies also to panel C, but the curves in this panel were fitted to the results obtained in all the repetitions. *trk1 trk2* cells exhibit a low affinity uptake of Rb^+ or Na^+ [5] (for Na^+ the K_m is 100 mM and the V_{max} 10 nmol mg^{-1} min^{-1}, and for Rb^+ the K_m is 60 mM and the V_{max} 35 nmol mg^{-1}

min^{-1}), which is not suppressed in pRH22 transformed cells. To calculate the kinetic parameters reported in Table 3, the rates mediated by the low affinity component have been ignored (this is especially important for Na^+) fitting the experimental data points to the addition of two Michaelis–Menten equations and reporting the parameters of the component with the highest affinity. For fitting the experimental data to the addition of two Michaelis–Menten equations or to Eq. (1), the "GraFit Scientific Graphs and Curve Fitting" (Leatherbarrow, R.J. 2001. GraFit version 5, Erithacus Software, Horley, UK) was used. Because of the problem of possible convergence to a local minimum of least squares solutions, tentative parameters were always obtained by a graphic approach (Eadie–Hofstee or double reciprocal plots) and used as initial estimates. In the case of Eq. (1), several fittings were performed in the whole range and partial ranges of concentrations.

3. Results

3.1. ScTRK1 mediates "active" K^+ uptake

The experiments to be described in this report were always carried out on a *trk1 trk2* disruption mutant [9] transformed with a centromeric plasmid containing the *ScTRK1* gene, which included 484 bp upstream of the first ATG in the open reading frame encoding the TRK1 transporter. The transformed strain grew like a wild-type strain at low K^+, and the kinetics of Rb^+ influx was identical to the wild-type kinetics, which deviates from a Michaelis–Menten kinetics and produces convex Eadie–Hofstee plots at low Rb^+ [13,14]. However, unlike a wild strain, our transformed strain also exhibited a low-affinity kinetic component (see kinetic constants in Materials and methods section), which was the ectopic Rb^+ influx of *trk1 trk2* mutants that results from the hyperpolarization state of these mutants [5]. All this indicates that the *TRK1* gene suppresses the K^+ uptake defect of the *trk1 trk2* mutant but not its abnormal hyperpolarization, the suppression of which probably requires the concourse of *TRK2*.

The first question about ScTRK1 is whether it mediates "active" K^+ uptake [1]. To answer this question we studied the effect of the protonophore uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on ScTRK1- or AtAKT1-mediated K^+ uptake. K^+-starved cells of the *trk1 trk2* mutant strain expressing either AtAKT1 or ScTRK1 depleted external K^+ down to 1–2 μM (Fig. 1). AtAKT1 is a channel [15], and was dramatically affected by CCCP. At 40 μM CCCP, the AtAKT1 cells could not deplete K^+ below 12 μM, and at 80 μM the cells lost K^+. This demonstrated a notable depolarization of the plasma membrane, especially at 80 μM CCCP. In contrast, ScTRK1 cells were much less affected by CCCP, and in the presence of 80 μM CCCP, the cells still depleted K^+ down to 20 μM (Fig. 1). To analyze the effect of CCCP in the

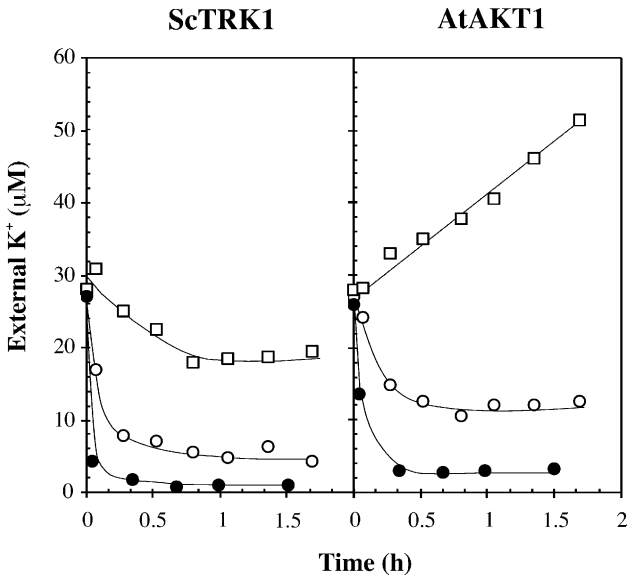

Fig. 1. Effect of CCCP on the capacity of the ScTRK1 transporter and the AtAKT1 K^+ channel to deplete external K^+. K^+-starved cells of the *trk1 trk2* mutant expressing either ScTRK1 or AtAKT1 were suspended in testing buffer pH 7.2 in the absence of CCCP (close circles) or in the presence of 40 (open circles) or 80 (open squares) μM CCCP. Then external K^+ was increased up to 30 μM.

TRK1 expressing cells, it has to be considered that Rb^+ influx in yeast, which is calculated as the initial rate of uptake (zero-trans conditions), is inhibited by 80 μM CCCP [12]. This suggests that the rise in the steady state of maximal K^+ depletion in the presence of CCCP is probably a kinetic consequence of the influx decrease, and does not reflect the change of the thermodynamic equilibrium. This response can be expected if ScTRK1 is either a symporter, which is working far from equilibrium (see the energetics of a symporter in [16]), or if K^+ uptake is coupled to a source of chemical energy. A symport with Ca^{2+}, which is the only abundant cation in the buffer, could be ruled out because K^+ uptake was not affected by EGTA (experiments like the control experiment in Fig. 1 but with EGTA). Similarly, experiments that rule out that ScTRK1 is an Na^+–K^+ symport are described below.

The possibility that ScTRK1 is an H^+–K^+ symporter has been proposed repeatedly with weak experimental support [1]. Although this possibility must be considered until the mechanism of ScTRK1 is established, the absolute independence of Rb^+ influx from the external pH in the 4.0 to 8.0 range [12] would be surprising for a K^+–H^+ symport. Now we have tested the effect of pH 8.0 on K^+ depletion, once again finding no pH effect (experiments like the control experiment in Fig. 1 but at pH 8.0).

3.2. Rb^+ influx in ScTRK1 can be modeled as an Rb^+–Rb^+ symport

We have already explained that the kinetics of ScTRK1-mediated Rb^+ influx deviates from the Michaelis–Menten

kinetics that is normally exhibited by a uniporter with a single cation binding site, and our next step was to investigate whether ScTRK1 cotransports two identical ions [1]. The kinetic properties of an Rb^+–Rb^+ symporter can be described by means of a 6-state reaction model (Fig. 2A), which can be simplified to a pseudo 3-state model (see Ref. [17]), lumping together many steps that are out of experimental control. In zero-trans conditions (we always record the initial rate of uptake, and at the beginning of the uptake experiment there is not Rb^+ inside the cells), if the total carrier amounts to N molecules, the concentrations of the three states of the carrier, N_1, N_2 and N_3 (Fig. 2B), can be solved when the carriers reach the steady state (see Ref. [18]). Then, the Rb^+ influx ($v = N_3 k_{31}$) is described by Eq. (1)

$$v = \frac{V_{max}[Rb^+]^2}{[Rb^+]^2 + K_1[Rb^+] + K_2} \quad (1)$$

in which

$$V_{max} = k_{31}N \quad K_1 = \frac{k_{31}}{k_{12}} + \frac{k_{32}}{k_{23}} + \frac{k_{31}}{k_{k23}}$$

$$K_2 = \frac{k_{21}}{k_{12}}\left(\frac{k_{32}}{k_{23}} + \frac{k_{31}}{k_{23}}\right).$$

Examination of Eq. (1) reveals that, at sufficiently low Rb^+ concentrations, the terms in the denominator containing $[Rb^+]$ are insignificant versus K_2, and the rate approaches a second order kinetics. On the contrary, above a certain $[Rb^+]$ value, K_2 becomes insignificant versus the terms containing $[Rb^+]$, and the influx approaches a Michaelis–Menten kinetics (then the K_m is the K_1 of Eq. (1)). This is the observed kinetics in all TRK transporters (see Ref. [12]) if the experiments are not specifically addressed to test the rates at low Rb^+ concentrations.

In baker and brewing yeast, the deviation of K^+ or Rb^+ influxes from a Michaelis–Menten kinetics were described many years ago in cells starved in water for several hours, and explained by the existence of two cation-binding sites, a transport site and an activation site (see a discussion of this hypothesis in Ref. [8]). The use of yeast cells starved in water has a technical advantage because they exhibit K^+ and Rb^+ K_m's that are higher than in yeast starved in growing medium [12], but there is a problem, because these cells exhibit nonphysiological exchanges of ions [19]. In physiological conditions, the high-affinity K^+ transporter of yeast cells can be studied in two states, high-affinity and medium affinity, 0.2 and 0.7–0.5 mM Rb^+ K_m's, and it has been described how medium affinity transforms into high affinity during K^+ starvation [13]. In the high-affinity state, it is technically difficult to generate enough influx data to carry out a kinetic study of the deviation that takes place at low cation concentrations, if $^{42}K^+$ is not available and Rb^+ is used in the study. The problem is that, in a K^+-free buffer, yeast cells lose K^+ and they cannot be kept in less than 0.5–1.5 μM external K^+ (Fig. 1), which interferes with the binding of Rb^+ to the first binding site. Therefore, our first kinetic approach to answering the question of whether ScTRK1 cotranspots two Rb^+ per cycle of the transporter was addressed in azide-treated cells, in which the transporter is in medium affinity and the low concentration of the external K^+ produces a lower interference [13].

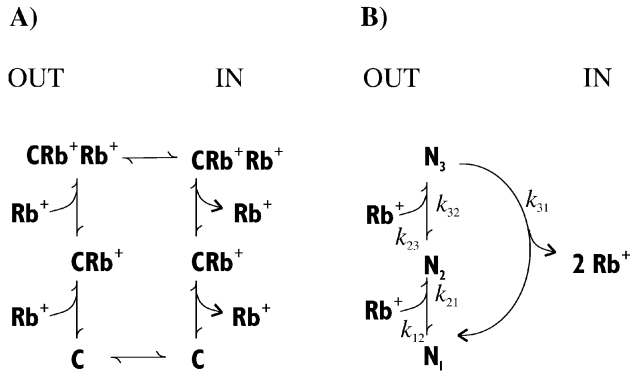

Fig. 2. Possible model for ScTRK1-mediated Rb^+ transport. (A) Explicit 6-state scheme. (B) Pseudo 3-state model in which several internal steps have been lumped together. k_{31} is also a lumped constant.

In our strain, Rb^+ influx mediated exclusively by ScTRK1 followed exactly the kinetics described previously for wild-type cells, which are furnished with TRK1 and TRK2, both in K^+-starved cells and azide-treated cells (Fig. 3A and B are Eddie–Hofstee plots for K^+-starved cells and azide-treated cells, respectively, and Fig. 3C is a semilogarithmic plot for azide-treated cells; the semilogarithmic plot was used to display in only one graph the complete concentration dependence of Rb^+ influx, which takes place in a range of Rb^+ concentrations of three orders of magnitude). The experimental data points in azide-treated cells could be fitted to Eq. (1) (see below and Table 2). This indicates that the observed kinetics is consistent with the model of a transporter with two transport sites.

The concentration dependence of Na^+ influx did not follow Eq. (1) or a simple Michaelis–Menten kinetics, either in azide-treated or K^+-starved cells. An extensive kinetic analysis of Na^+ influx in K^+-starved cells demonstrated that the kinetic was complex, due to the low-affinity ectopic uptake present in *trk1 trk2* mutants [5]. However, this kinetic component was clearly distinguished by mathematical data analysis (see Materials and methods) from that dependent on ScTRK1, which followed a Michaelis–Menten equation with a K_m of 14 mM and a V_{max} of 18 nmol mg^{-1} min^{-1}, if the kinetic study was restricted to Na^+ concentrations above 5 mM, approximately. At very low Na^+ concentrations, between 10 and 200 μM, the rates were again higher than those predicted by the Michaelis–Menten kinetics. This response can be explained taking into account the presence of K^+ (1–2 μM) in the buffer, and that this K^+ concentration enhances Na^+ influx at low Na^+ concentrations. We describe below that low Rb^+ concen-

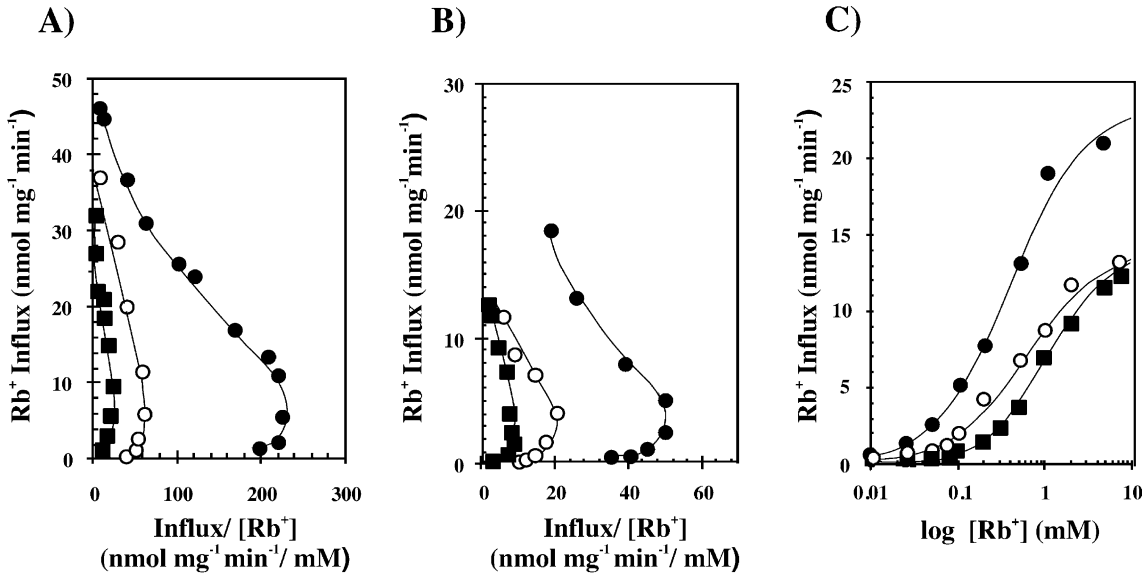

Fig. 3. Kinetic response of Rb^+ influx for the ScTRK1 transporter, and Q90R and L949P mutants. (A) Eadie–Hofstee plot of K^+-starved cells. (B) Eadie–Hofstee plot of azide-treated cells. (C) Semilogarithmic plot of the influx concentration dependence in azide-treated cells; the curves are plots of Eq. (1) with the parameters summarized in Table 2. Wild transporter (close circles); Q90R (close squares); L949P (open circles).

trations dramatically enhances Na^+ influx (Table 1) and we propose here that K^+ produces a similar effect. Because of the K^+ presence, our data did not allow to calculate the Na^+ K_2 parameter in Eq. (1).

3.3. ScTRK1 cotransports Rb^+ and Na^+ in special conditions

The perfect fit of the kinetics of the ScTRK1-mediated Rb^+ influx to Eq. (1) was a necessary condition but not a sufficient one to demonstrate Rb^+–Rb^+ cotransport, because a concentration dependence response like that shown in Fig. 3 can be also expected if the two-site transporter had activation and transport sites [8]. Therefore, we now address the question of whether ScTRK1 can transport two different ions simultaneously, because this would demonstrate that the two binding sites of the transporter are transport sites. At low K^+, as in Fig. 1, we found that K^+ was taken up alone, because in experiments carried out in a low Na^+ medium (<4 μM), the depletion of 30 μM K^+ did not consume or depend on Na^+. Similar experiments using Rb^+ also failed to demonstrate that Rb^+ influx depended on Na^+. However, when both Rb^+ and Na^+ were tested at 20–30 μM (experiments at pH 6.0 and 8.0), Na^+ did not enhance Rb^+ influx, but Rb^+ did enhance Na^+ influx. Similarly, when the influxes were tested with concentrations in the millimolar range for Na^+ and in the micromolar range for Rb^+, we could not observe Na^+ enhancement of Rb^+ influx, but Rb^+ enhanced Na^+ influx (Table 1).

These results clearly supported that ScTRK1 was not a typical K^+–Na^+ symporter, but two patterns of response of the cation influxes recorded in Table 1 did support that both ions were transported together in some conditions: first, 0.5 mM Na^+ inhibited Rb^+ influx by 40% at 0.05 mM Rb^+, but almost nothing at 0.2 mM Rb^+; and second, 0.05 mM Rb^+ enhanced threefold Na^+ influx at 0.5–2.0 mM Na^+, and slightly less at higher Rb^+ concentrations up to 0.5 mM.

A formal analysis of a model for the transport of Rb^+ and Na^+, as in Fig. 2, at all possible concentrations of both cations is very complex (it can be analyzed as a pseudo 7-state model). However, both the inhibition of Rb^+ influx by Na^+ and the enhancement of Na^+ influx by Rb^+, as described above, can be easily explained without a formal analysis of the model, taking into account the Rb^+ kinetic constants recorded in Table 2. In the case of inhibition by Na^+, at 0.05 mM Rb^+ the first site is almost saturated and the second is responding to increasing concentrations (influx of 10 versus a V_{max} of 38 nmol mg^{-1} min^{-1}). Then, the addition of 0.5 mM Na^+ cannot have any effect

Table 1
Interactions between the Rb^+ and Na^+ influxes mediated by ScTRK1

Rb^+	Na^+ added					
	0 mM		0.5 mM		2 mM	
	Rb^+	Na^+	Rb^+	Na^+	Rb^+	Na^+
mM	nmol mg^{-1} min^{-1}					
0				1.4		2.7
0.05	10		6	4.1	6	5.6
0.2	24		23	3.0	16	5.6
0.5	31		33	2.8	27	3.0

K^+-starved cells of the *trk1 trk2* mutants expressing ScTRK1 were suspended in testing buffer pH 6.0 and exposed to different Rb^+ and Na^+ concentrations. The initial rates of uptake (influxes) were calculated from the time courses of Rb^+ and Na^+ accumulations.

Table 2
Summary of kinetic analyses of ScTRK1-mediated Rb^+ influx

Strains	K^+-starved cells			Azide-treated cells		
	V_{max} (nmol mg^{-1} min^{-1})	K_1 (mM)	K_2 (mM)	V_{max} (nmol mg^{-1} min^{-1})	K_1 (mM)	K_2 (mM)
WT	38	0.12	[a]	25	0.41	0.002[b]
Q90R	25	0.45	0.200	15	1.04	0.190
L949P	38	0.62	0.003	14	0.63	0.008

The initial rates of Rb^+ uptake in two types of cells expressing the wild transporter or the two mutants were fitted to Eq. (1); the fits in azide-treated cells are shown in Fig. 3C.

[a] This parameter cannot be calculated because of the contaminant K^+.

[b] This value is higher that the real parameter because of the contaminant K^+.

on the second binding site, because of the low affinity of Na^+ for this site (a K_m of 14 mM in Table 3), but it will displace most of the Rb^+ (0.05 mM) from the first site, if the Rb^+ and Na^+ affinities for this site are not very different. As a consequence, the influx will decrease to one half because, in most of the cycles, the transporter would carry out Na^+ and Rb^+ instead of two Rb^+. The model also predicts that a further, modest increase of Na^+ will affect very little the Rb^+ influx, because Na^+ is already saturating the first site and the concentration is still too low to displace Rb^+ from the second site. All this is consistent with the experimental data recorded in Table 1.

The enhancement of Na^+ influx by low Rb^+ concentrations can be explained in a similar way. At 0.5 mM Na^+, the influx is limited by the low affinity of Na^+ for the second site (the first is almost saturated). In these conditions, the addition of 0.05 mM Rb^+ will increase Na^+ influx, because Rb^+ binds to the second site and a high number of transporters will have now both sites occupied and can complete the cycle across the membrane. Although only one Na^+ is carried out in each cycle, the higher number of cycles compensates for the presence of only one Na^+ in the transporter. At higher Rb^+ concentrations, Rb^+ will displace Na^+ from the first site and inhibit Na^+ influx.

3.4. Point mutations modifying the kinetics of the transporter

To understand the function of TRK1 better, we isolated a collection of mutants (see Materials and methods). Two of them carried out one-amino-acid changes, Q90R and L949P, which dramatically affected the kinetics of Rb^+ influx. Fig. 3 shows the effect of the mutations on this kinetics, and Table 2 summarizes the calculated parameters after fitting the experimental data to Eq. (1). The effect of the Q90R mutation was to increase more than 100-fold the K_2 constant, producing less than a threefold increase in K_1, and to decrease slightly the V_{max} (Table 3). The consequence of the dramatic increase in K_2 was that the concentration dependence of the influx deviated from a Michaelis–Menten kinetics even at concentrations higher than K_1, because

Table 3
Kinetic parameters of ScTRK1-mediated Rb^+, Na^+ and K^+ transport in K^+-starved cells

Strains	Rb^+		Na^+		K^+
	K_m (mM)[a]	V_{max} (nmol mg^{-1} min^{-1})	K_m (mM)	V_{max} (nmol mg^{-1} min^{-1})	K_m (mM)[b]
WT	0.12	38	14	18	0.014
Q90R[c]	0.45	25	4	2	0.4
L949P	0.62	38	4	2.5	0.16

The initial rates of Rb^+ or Na^+ uptake in K^+-starved cells expressing the wild transporter and the two mutants were calculated at Rb^+ and Na^+ concentrations, for which deviations from a Michaelis–Menten kinetics are negligible.

[a] This is the K_1 constant of Table 2.

[b] Calculated as the K_i of K^+ over Rb^+ influx.

[c] In this mutant Rb^+ influx deviates significantly from a Michaelis–Menten kinetics, at 1 mM Rb^+ the deviation is 15%.

when K_2 was not much lower than K_1, Eq. (1) never resembled a Michaelis–Menten equation (however, in Table 3 we kept the term K_m in this mutant for simplification). The effect of L949P was mild for both K_1 and K_2, and its most remarkable effect was to abolish the decrease of K_1 that occurs when the cells are K^+-starved.

To further investigate the effect of these mutations we studied their effects on K^+ and Na^+ transport. For K^+, we tested the K^+ inhibition of Rb^+ influx, because $^{42}K^+$ is not available to us and it has been demonstrated that K^+ and Rb^+ compete for transport, and that the K^+ K_m is equal to the K_i of K^+ upon Rb^+ influx [12]. These experiments revealed two remarkable effects of the mutations (Table 3), that they decreased the Na^+ K_m, the opposite of the effect on Rb^+ influx, and that the K^+ K_m increased much more than the Rb^+ K_m. In both mutations, the affinities of the alkaline cations for the transporter became close in value, especially in Q90R, which did not discriminate between K^+ and Rb^+, and exhibited only a 10-fold ratio between the K^+ and Na^+ apparent K_m's (1000-fold in the wild type). Interestingly, although the mutations increased the affinity of the transporter for Na^+, they decreased dramatically its Na^+ transport capacity, because they decreased the V_{max}'s, ninefold in Q90R and sevenfold in L949P.

Table 4
Interactions between the Rb^+ and Na^+ influxes in the Q90R mutant

Rb^+ added	Na^+ added					
	0 mM		2.0 mM		5.0 mM	
	Rb^+	Na^+	Rb^+	Na^+	Rb^+	Na^+
mM	nmol mg^{-1} min^{-1}					
0				0.5		1.0
0.1	1.0		0.4	0.7	0.4	1.3
0.2	2.8		1.0	0.7	0.9	1.4
0.5	9.5		3.2	1.4	2.4	2.2
1	15		7.4	1.5	4.8	2.3
2	18		14	1.3	10	2.7

Conditions as described for Table 1, except that the Q90R mutant was used.

Table 5
Interactions between the Rb^+ and Na^+ influxes in the L949P mutant

Rb^+	Na^+ added					
	0 mM		2.0 mM		5.0 mM	
	Rb^+	Na^+	Rb^+	Na^+	Rb^+	Na^+
mM	nmol mg^{-1} min^{-1}					
0				1.0		1.7
0.1	7		4.5	2.7	3.4	3.4
0.2	12		8.0	3.2	6.4	4.8
0.5	21		15	3.2	13	5.7
1.0	30		21	2.3	20	4.8

Conditions as described for Table 1, except that the L949P mutant was used.

As described for the wild-type transporter, the mutants also took up Rb^+ and Na^+ together (Tables 4 and 5), and all the interactions explained for the wild transporter can be applied to the mutants. Rb^+ enhanced Na^+ influx and Na^+ inhibited Rb^+ influx, but the quantitative effects were affected by the described changes in the kinetic parameters. For example, in the mutants, Na^+ was more inhibitory on Rb^+ influx because the competitive effect of Na^+ on the second site increased with the decrease of Na^+ K_m in the mutants, 4 versus 14 mM (see the effect of 2 mM Na^+ over the influx at 0.5 mM Rb^+). Furthermore, in the Q90R mutant, the decrease of the Rb^+ V_{max} and especially the Na^+ V_{max}, indicates that the turnover rate of the transporter has decreased markedly when it carries Na^+ and, according to the results reported in Table 4, also when it carries Rb^+ and Na^+. In the L949P mutant the Na^+ V_{max} decreased sevenfold, but Rb^+ V_{max} was not affected. In this mutant Na^+ inhibited Rb^+ influx by 35% (Table 5), which suggests that the turnover rate of the transporter is close to normal when it carries Rb^+ and Na^+.

4. Discussion

The ScTRK1 K^+ transporter is perfectly suited to mediate K^+ uptake in accordance with the K^+ requirements of the yeast cells under any circumstances. Its K^+ K_m is millimolar when the external K^+ is millimolar [12], and it can decrease continuously down to 0.05 mM by an unknown mechanism of kinetic control, when the cells detect a modest decrease in K^+ content. If the cells suffer a drastic decrease in their K^+ content, the transporter may decrease the K^+ K_m further, down to 0.02 mM (this is an all-or-nothing process during which each transporter may be in the 0.05- or 0.02-K_m state) by a physical transformation of the transporter which takes 3 h to be completed in a wild-type strain [13]. Our finding that at micromolar K^+ concentrations K^+ uptake is active (Fig. 1) adds an interesting feature to this extraordinary transporter. Although we could not characterize the coupling mechanism, the pH independence of the process suggests that a K^+–H^+ symport is unlikely, as we discuss below.

ScTRK1 belongs to a large family of cation symporters that have evolved by successive duplications of an ancestor of the KcsA channel, many of which function as K^+–Na^+ symporters [2,20]. In accordance with this model we propose that ScTRK1 has two transport sites (Fig. 2) and that its kinetic response is explained by Eq. (1). However, because the K_2 values for K^+, Rb^+ and Na^+ are much lower than the K_1 values, the kinetics of K^+, Rb^+ and Na^+ influxes in K^+-starved yeast cells follow a Michaelis–Menten equation [12,21], if the concentration dependence of the influxes is not tested at concentrations much lower than the apparent K_m's.

The K^+ transporter of *S. cerevisiae* was described long ago as a two-site transporter (see also proposals of a third site in Ref. [8]), for which the concentration dependence of influx was described by a quadratic rate equation [14] very similar to Eq. (1). Although in a kinetics of this type it is difficult to distinguish whether the two sites participate in transport, the hypothesis that one of them plays only an activation function was established in the first reports, because Ca^{2+} and other cations activated transport but were not transported [22,23]. Surprisingly, this hypothesis has not been questioned in more recent reports. However, at present the support by the Ca^{2+} results is weak, because it is known that Ca^{2+} is required for physiological activity of fungal K^+ transporters and that in its absence their functions may be seriously impaired, depending on the transporter and the testing conditions [19,24]. Considering all these circumstances and that present results are essentially coincident with previous ones, the two-site transport mechanism for ScTRK1 is out of the question, and the only question at present is whether the two sites participate in transport.

We have already explained in Results that the two transport site hypothesis explains the reciprocal effects of Na^+ and Rb^+ on their respective influxes: that Na^+ concentrations much lower than the K_m can inhibit Rb^+ influx, and that low Rb^+ concentrations enhances Na^+ influx. On the contrary, the former effect cannot be explained by the activation site model, unless the activation site transforms into an inhibitory site when it binds Na^+, which is inconsistent even with the results that gave rise to the hypothesis of the activation site [14]. Regarding the activation of Na^+ influx at low Na^+ concentration by 10-fold lower Rb^+ concentrations (Table 1), it is unnecessary to invoke the existence of an activation of first-site binding to explain it, because it is the consequence of the existence of two transport sites, when the second one has a much higher affinity for Rb^+ than for Na^+. The only condition is that the affinities of the first site for Rb^+ and Na^+ are not very different, as proposed in previous reports, which reported apparent dissociation constants of 0.020 and 0.032 mM for Rb^+ and Na^+, respectively [8]. To sum up, the two transport site model is consistent with previous and present experimental data, but the activation and transport site model cannot explain a part of present results.

As a physical proof of the cotransport model, we tried to measure "active" K^+ uptake driven by Rb^+ influx in uncoupled cells. Although the test seemed to be possible, because ScTRK1 bound both cations with high affinity, the test discovered a remarkable characteristic of ScTRK1, because in CCCP uncoupled cells it still mediated an "active" process (Fig. 1). Present results can rule out that "active" K^+ uptake mediated by ScTRK1 is driven by the Na^+ gradient, because Na^+ is not taken up with K^+. A K^+–H^+ symport [25,26] may be the mechanism involved, but it is unlikely, considering the insensitivity of Rb^+ influx [12] and K^+ uptake (here described) to high external pH. Obviously, if H^+ bound the transporter with very low "apparent" K_m, for example 1 nM (half rate at pH 9), the effect of the pH would be technically undetectable. However, this seems unlikely in an organism such as *S. cerevisiae* that is adapted to low-pH media. Therefore, although the mechanism of the active K^+ uptake mediated by ScTRK1 cannot be predicted at the moment, the possibility that ScTRK1 couples chemical energy is attractive.

The analysis of the two mutants that we reported, Q90R and L949P, gave further support to the proposed model and revealed some properties of the transporter. The Q90R mutation increased the Rb^+ K_2 100-fold and the Rb^+ K_1 threefold (Table 2). Although according to these results, Gln^{90} seems to be involved only in the protein structure that determines K_2, that conclusion does not hold when the effects on K^+ influx are considered, because in this case the K_1 increased 30-fold (the K_m in Table 3). In contrast with the decrease in the affinity of the Q90R transporter mutant for K^+ and Rb^+, the mutant transporter exhibited a higher affinity for Na^+. These results suggest that the two transport sites of ScTRK1 may be physically coincident with slightly different binding amino acids or, if they are not coincident, they are very close. Otherwise, one-amino-acid change should not affect the two binding constants in such a specific way. This conclusion might also hold for K^+–Na^+ symporters of the same family, such as HKT1 from wheat or KtrB from *Vibrio alginolyticus*, because the physical structure of all the transporters of this family is very similar.

According to the tetra-MPM model of Ktr and TRK transporters [2,20], Gln^{90} is situated in the link between the P_{1A} and P_{2A} helixes (Fig. 4) close to P_{2A}, which in most channels forms the selectivity filter in the entrance of the pore. In the KcsA channel the selectivity filter is formed by the amino acid sequence Val–Gly–Tyr–Gly [6], and in ScTRK1 the corresponding sequence is Thr^{91}–Gly–Leu–Asn [2], immediately after Gln^{90}. Therefore, the loss of selectivity of Q90R can be considered the consequence of a change in the selectivity filter, as those described previously for the AtKAT1 channel [27] and for the KtrB K^+–Na^+ symporter of *V. alginolyticus* [7]. In addition to its effects on the selectivity of the transporter, decreasing the affinity for K^+ and Rb^+ and increasing the affinity for Na^+, Q90R affected the rate of transport, decreasing ninefold the Na^+ influx V_{max} but only 1.5-fold the Rb^+ influx V_{max} (Table 3). Taken together, the physical model and the effects of the Q90R mutation suggest that the two binding sites for K^+ are close to Gln^{90}, in the entrance of the pore, which in the most likely models is involved in the binding of the two symported cations [20].

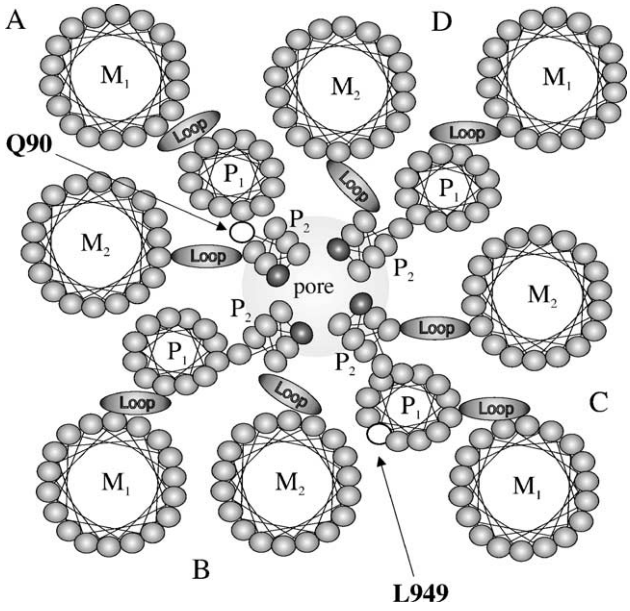

Fig. 4. A schematic model of the ScTRK1 transporter and position of the Q90R and L949P mutations. Model based on Refs. [2,20]. The fragments connecting the four $M_1P_1P_2M_2$ elements, A, B, C and D, have been omitted. The conserved glycine residue in each P_2 element is shown as a darker sphere.

In the physical model of ScTRK1, L949 is situated in the P_{1C}-loop helix (Fig. 4), outside the pore. Therefore, it is not surprising that the L949P mutation did not have a strong effect on the kinetics of the transporter, producing only a fivefold increase in the Rb^+ K_2, no effect on Rb^+ K_1, a 10-fold increase in K^+ K_1 and a threefold decrease in Na^+ K_1. This involves again a decrease in the selectivity of the transporter, an effect that must be added to the most striking one of suppressing the transformation of the transporter from the medium to the high affinity state (Table 2). This change occurs when the cells are K^+-starved [13], but also when they are under Na^+ stress, because it involves an increase in the cation selectivity of the transporter and this increases Na^+ tolerance [28]. Considering that the change from medium to high affinity probably involves a physical change in the transporter [13], and that the introduction of a proline in the P_{1C}-loop helix should produce an important physical disturbance in the protein around the mutation, a likely hypothesis is that the P_{1C}-loop helix interacts in some way with the selectivity filter of ScTRK1. In the wild-type transporter, this loop may transmit to the selectivity filter the interaction with another protein or the effect of a phosphorylation/dephosphorylation process. In the mutant, it seems that the misshapen loop is insensitive to the regulatory

process, and that the transporter is blocked in the medium affinity state or close to it.

The evolution of fungal TRK transporters to become K^+–K^+ symporters (K^+ uniporters) from an ancestral K^+–Na^+ symporter is consistent with the evolution of fungi, which associated with plants that took part in the conquer of the lands that emerged from the sea some 450 million years ago [29–31]. The poor rocky environment conquered by the fungal-plant association early in the Paleozoic era had a low Na^+ concentration, and although the medium changed by the effect of the biological activity [32,33], the association evolved for a long time in an oligotrophic soil in which the Na^+ content was low [34,35]. This medium was common for plants and fungi, and both adapted to it using the same H^+-pump ATPase [36] and similar K^+ transporters [1]. However, even in this low Na^+ medium, plants may need transporters to remove Na^+ from the xylem sap when Na^+ is present, and this is a function that can be fulfilled by an HKT transporter evolved from an ancestral Ktr transporter. This would explain the great variability of plant HKT transporters, which transport K^+ and Na^+ or only Na^+ [37–39]. In contrast, fungal transporters evolved to become K^+-specific. In addition to ScTRK1, five other fungal TRK transporters have been studied, from *S. cerevisiae* [4], *N. crassa* [9], *S. occidentalis* [40] and *S. pombe* [26,41]. As for ScTRK1, the kinetic analyses and the experimental conditions in which the corresponding assays were carried out suggest that all these transporters mediate the uptake of only K^+ (or Rb^+) and are not K^+–Na^+ symporters [1]. A more intense research on these transporters, to clarify the driving force involved and number of cation binding sites, will help to understand the functioning and evolution of the TRK-HKT family of transporters

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