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Functional analysis of the $M2_D$ helix of the TRK1 potassium transporter of *Saccharomyces cerevisiae*

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Abstract

Eukaryotic KcsA-related K^+ transporters mediate physiologically relevant K^+ and Na^+ fluxes in fungi and plants. ScTRK1 is a characteristic member of the group, and here we report a mutational analysis of the unique $M2_D$ helix of this transporter. Our results support the theoretical models placing this helix in a relevant position in the pore and interacting with P segments. Most single mutations eliminating positively charged or introducing negatively charged residues reduced the V_{max} of Rb^+ influx to a half, several together showed an additive effect, and four practically suppressed transport. In contrast, the introduction of only one positively charged residue practically abolished the function of the transporter. Almost all mutations in the $M2_D$ helix affected the two Rb^+ binding sites of the transporter, mimicking mutations in the selectivity filter.
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1. Introduction

Transport of cations through biological membranes is mediated by channels and several types of pumps and carriers. In eukaryotic non-animal cells, most of the K^+ and Na^+ influx carriers belong to the TRK-HKT and HAK transporter families, in which there are also bacterial carriers. The TRK and HKT transporters, which are probably universally present in fungi and plants, are related to many channels because the members of this family have evolved from an ancestor close to the prokaryote KcsA channel of *Streptomyces lividans* [2,3]. In fungi, TRK transporters play more functions than the mere K^+ uptake [4–6] and, in plants, HKT transporters have especial importance because they provide a pathway for Na^+ entrance and contribute to Na^+ exclusion from the shoots [7–9].

KcsA-related K^+ and Na^+ transporters share a common structure of four M1PM2 (4 × two transmembrane α-helices connected by the roughly 30-amino-acid P segment) motifs grouped in a single sequence [2,3] in which some amino acid residues are conserved in the repeated motifs. Because maximum conservation occurs in the important P loops, which form the inner part of the transporter and share properties with the selectivity filter of channels, most of the structural–functional studies have addressed these P loops [10–12]. However, a fuller understanding of the function of the KcsA-related transporters, many of which cotransport two cations, goes further than the understanding of the selectivity filter and refers to their physical structure. This has been rigorously modeled [2], but no study has been addressed to give empirical support to the model.

In the structure of the TRK and HKT transporters the $M2_D$ helix is unique. It shows the highest sequence conservation after P segments and presents a notorious accumulation of positively charged amino acid residues. This helix has a lower number of charges in prokaryotic transporters, but many of them share two charged amino acids with eukaryotic transporters. The rather polar structure of the $M2_D$ helix can be explained by a tetramer model of the transporter (four TRK proteins) in which polar residues are not greatly exposed to the lipid alkyl chains, and the positively charged residues are electrically balanced by the formation of salt bridges with negatively charged residues in

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other transmembrane helices or in P segments. This model implies that $M2_D$ forms part of the pore and interacts with the selectivity filter, a prediction that can be functionally tested.

The ScTRK1 transporter of *Saccharomyces cerevisiae* is the eukaryotic member of the TRK-HKT family of transporters in which more functional research has been carried out. Both the regulation of its transport kinetics and the functional consequences of its gene deletion have been deeply investigated [1]. Recently, it has been proposed that ScTRK1 has two K^+ or Rb^+ binding sites involved in transport and that the two binding constants involved in its two cation transport kinetics are affected by mutations in the P_A fragment [11]. To continue the research on ScTRK1 and on the structural–functional relationships of TRK-HKT transporters, we have studied the functional consequences of both eliminating positively charged residues and introducing negatively or positively charged residues in the $M2_D$ helix of the ScTRK1 transporter. We report here that the results of these experiments support partially the theoretical model [2].

2. Materials and methods

2.1. Strains, plasmids, and growth conditions

The *Escherichia coli* strain DH5α was routinely used for plasmidic DNA propagation. The *S. cerevisiae* strain WΔ6 was specifically constructed for this report deleting the complete coding regions of the *TRK1* and *TRK2* genes of strain W303.1A (*Mata ade2 ura3 leu2 his3 trp1*). The disruptions were carried out by homologue recombination of fragments containing a marker gene, *LEU2* for *TRK1* and *HIS3* for *TRK2*, and flanking homology regions of each gene, from positions −117 to −167 and 3570 to 3620 in *TRK1* and from −165 to −204 and 2719 to 2768 in *TRK2*, which were constructed by PCR [13]. The TRK1 transporter was expressed from plasmid pTRK24, a pFL38-based [14] centromeric-plasmid into which the *ScTRK1* gene was transferred from plasmid pRH22 [15]. Yeast transformants were routinely grown in SD medium [16] and *trk1 trk2* mutants in the same medium supplemented with 25 mM K^+, for some purposes cells were grown in arginine phosphate medium (AP medium) [17]. Na-azide treated cells were obtained by exposing actively growing cells to 10 mM Na-azide in K^+-free AP medium for 10 min [11,18].

2.2. DNA manipulations and mutant constructions

Manipulation of nucleic acids was performed by standard protocols or, when appropriate, according to reagent manufacturer's instructions. Mutants were constructed on plasmid pTRK24, by PCR-based targeted mutagenesis [19]. All constructs were sequenced by using an automated ABI PRISM 377 DNA sequencer (Applied Biosystems).

2.3. Transport assays

Na-azide treated cells or K^+-starved cells were suspended in testing buffer: 2% glucose, 10 mM MES brought to pH 6.0 with $Ca(OH)_2$, and, at intervals after the addition of Rb^+, Na^+, or Li^+, samples were taken, filtered through 0.8-μm-pore nitrocellulose membrane filters (Millipore) and washed with 20 mM $MgCl_2$ in the same filter. Filters were incubated overnight in 0.1 M HCl, and Rb^+, Na^+, or Li^+ was determined by atomic emission spectrophotometry of acid-extracted cells [11]. Data were analyzed as described previously. Data in Table 1 can be affected by an error of ±1 unit of the last reported figure. This does not apply to the quadruple mutant R1156S/K1158M/R1160I/N1159D and single mutant M1153R. In these mutants, the V_{max} is very low, 1–2 Rb^+ nmol mg^{-1} min^{-1}, and no fitting program can make a good fitting over the background of the WΔ6 intrinsic transport (40 Rb^+ nmol mg^{-1} min^{-1} of V_{max} and 58 mM Rb^+ K_m). For the kinetic analysis of transporters expressed in *trk1 trk2* mutants, a subtraction procedure is absolutely precluded because frequently the expression of a new transporter can modify the kinetics of the intrinsic K^+ or Rb^+ transport of these strains.

3. Results

3.1. Mutations in $M2_D$ affected K_m and V_{max} of Rb^+ influx

ScTRK1 is a typical KcsA-related eukaryotic transporter, except for the presence of a Lys^{1158} residue in the position where other eukaryotic transporters have an arginine residue (Fig. 1). The functional consequences of altering the $M2_D$ helix of ScTRK1 were studied using centromeric plasmids containing the wild-type or mutated versions of the *ScTRK1* gene, in all cases conserving the original promoter. These plasmids were transformed in a new *trk1Δtrk2Δ* strain, specifically constructed for this work, in which the entire coding regions of *TRK1* and *TRK2* were eliminated. Previous versions of *trk1 trk2* disruption mutants [20,21] can express the first M1PM2 motifs of the TRK1 and TRK2 transporters, and this may produce a certain grade of interference with expressed transporters (R. Haro and A. Rodríguez-Navarro, unpublished results). Table 1 summarizes the kinetic consequences of the mutations on Rb^+ influx and on its inhibition by Na^+, in cells that had been treated with Na-azide to eliminate 50% of their cellular K^+ content, and bring the transporter to a high-affinity state [11]. We record two kinetic constants for Rb^+ influx, K_1 and K_2, because ScTRK1 has two binding sites for Rb^+. In the wild-type transporter, the effect of K_2 in the kinetics is only detected at low Rb^+ concentrations and the customary K_m is really the K_1 constant [11]. To show graphically the effects of the mutations on the K_1 and K_2 constants, Fig. 2 shows the V_{max}-normalized Eadie–

Table 1
Effects of mutations in the $M2_D$ helix of the ScTRK1 transporter of *S. cerevisiae*

Transporter	Rb^+			Na^+
	V_{max} (nmol mg^{-1} min^{-1})	K_1 (mM)	K_2 (mM^2)	K_i (mM)
Wild-type transporter	21	0.38	0.0015	3
K1147N	14	1.4	0.055	6
R1156S	8.0	1.4	0.015	6
K1158M	9.0	0.94	0.0078	4
R1160I	21	0.38	0.0015	2
R1156S/K1158M/R1160I	3.7	1.7	n.d.	n.d.
N1159D	8.5	0.97	$<10^{-4}$	4
R1156S/K1158M/R1160I/N1159D	1–2	1–2	n.d.	n.d.
M1153R	1–2	0.4–1	n.d.	n.d.
M1153E	7.0	0.38	0.0082	1
M1153Q	10	1.1	0.0015	8

Kinetic constants of Rb^+ influx and Na^+ inhibition of Rb^+ influx in Na-azide treated cells.
The initial rates of Rb^+ uptake in *trk1trk2* yeast mutant expressing the wild ScTRK1 transporter and its mutants were fitted to equation $v = \frac{V_{max}[Rb^+]^2}{[Rb^+]^2 + K_1[Rb^+] + K_2}$ [11].

Hofstee plots (plots in which the effects of V_{max} differences have been eliminated by plotting v/V_{max} versus $(v/V_{max})/[Rb^+]$).

As a first analysis of the transporter, we replaced, one by one, the four positively charged amino acid residues by non-charged residues, finding that, with only one exception, R1160I, the elimination of charged residues affected the transport capacities of ScTRK1 (Table 1). Regarding their effects on the V_{max}, whose changes could reflect changes in the pathway across the pore, the three mutations, K1147N, R1156S, and K1158M, produced around a 2-fold decrease. The K_1 and K_2 constants, which probably reflect the binding characteristics of the transporter, both were affected by the mutations, but in independent ways. In the K1147N and R1156S mutations, the K_1's were identical, 3.7-fold larger than in the wild-type transporter, whereas the K_2's increased 37- and 10-fold, respectively. In the K1158M mutation, the increases were 2.5-fold for the K_1 and 5-fold for the K_2. Interestingly, when three of these mutations were put together, R1156S K1158M R1160I, the V_{max} of Rb^+ influx decreased 6-fold, but the K_1 increased very slightly above the value produced by a single mutation (1.7 versus 1.4 mM in R1156S). In this triple mutant, the low V_{max} impeded the determination of the K_2 constant by technical problems. However, it can be predicted that the K_2 was not high (<0.1 mM^2) because the kinetics of Rb^+ influx exhibited a Michaelis–Menten kinetics in the concentration range that we could test, approximately down to 3-fold below the K_m.

Our next test was to create a negative charge in the core of the positively charged residues, which is formed by the **RGKNR** sequence. For this purpose, we selected the asparagine residue, which is not conserved in other transporters of the family (Fig. 1C), and constructed the N1159D mutation. The effects of this mutation on the K_1 and V_{max} were almost identical to those produced by mutation K1158M, but unlike all the other mutations, it dramatically decreased the K_2 (Table 1). Formally, it decreased down to undetectable values, but this may be also interpreted as the lost of the first binding site of the transporter (see model in Ref. [11]). Addition of the N1159D mutation to the three-positive-charge defective mutant above described further decreased the V_{max} but probably did not affect the K_1 (the low V_{max} did not allow to calculate the K constants, as explained in Materials and methods section, but the data allow a certain prediction of the K_m, which is the K_1).

As a final test, the Met^{1153} residue situated approximately in the middle of $M2_D$ helix was mutated to charged or more

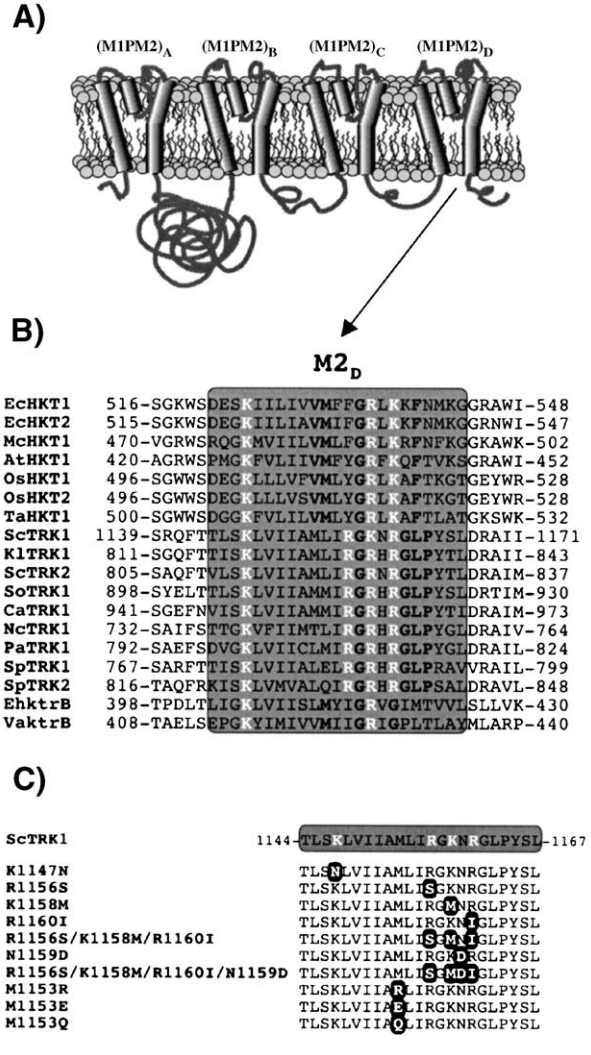

Fig. 1. Location of the mutations studied in the ScTRK1 transporter. (A) Schematic structural model of KscA-related transporters, eukaryotic TRK and HKT, and prokaryotes KtrB and TrkH transporters. (B) Sequence alignments of the $M2_D$ helix from eukaryotic and prokaryotic KcsA-related transporters, positively charged residues are highlighted in white. (C) Amino acid changes introduced in the $M2_D$ helix.

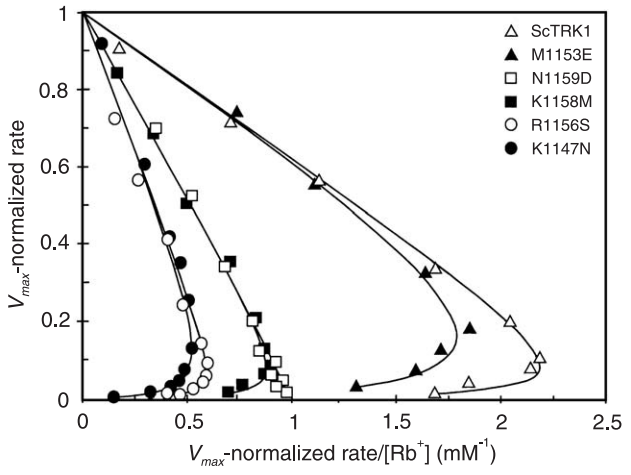

Fig. 2. V_{max}-normalized Eadie–Hofstee plots of the Rb^+ influx mediated by the wild-type ScTRK1 transporter and five mutants, K1147N, M1153E, R1156S, K1158M, and N1159D. To highlight the changes in K_1 and K_2, canceling the V_{max} effects, the rate at every Rb^+ concentration was divided by the corresponding V_{max}. Lines through experimental data points are equation plots that were obtained by applying the described transformation to the equation recorded in the footnote in Table 1 and the parameters recorded in the same table.

polar amino acids (M1153R, M1153E, M1153Q). In general terms, the two M1153E, M1153Q mutations produced similar effects to those described above for single mutations, but usefully demonstrating that the effects upon K_1 and K_2 could be dissociated (M1153E affected exclusively the K_2 and M1153Q exclusively the K_1). In contrast, the M1153R mutation abolished almost completely the transport capacity of ScTRK1 by making the V_{max} almost undetectable (despite the technical difficulties of testing a transporter with an insignificant V_{max}, our results suggest that the K_m was slightly affected).

In all the described mutations, we calculated the Na^+ K_i on Rb^+ influx to detect a possible change in the selectivity of the transporter (Na^+ produces a competitive inhibition at concentrations around the K_i value, but Na^+ influx and Na^+ inhibition at concentrations much higher or much lower than the K_i are very complex [11]). These tests revealed that the effects on the Na^+ K_i were small and correlated acceptably with the effects on Rb^+ K_1 (both the Rb^+ K_1 and Na^+ K_i increased, except in M1153E) (Table 1).

Growth tests at low K^+ carried out in parallel with the kinetic tests produced results consistent with the kinetic findings. Single mutations, except M1153R, had almost undetectable effects, whereas M1153R, triple, and quadruple mutants decreased notably the growth at low K^+ concentrations (e.g., at 0.5 mM K^+ in arginine phosphate medium).

3.2. Mutations in $M2_D$ did not decrease the number of transporters

The effects of the studied mutations on the K_1 and K_2 constants of Rb^+ influx very likely reflected functional changes of the protein, but this was not the case for the effects on the V_{max}. In this case, before drawing conclusions about functional effects, it was necessary to rule out that the mutations introduced in the $M2_D$ helix had decreased the number of transporters in the plasma membrane. This could be expected if the mutation had produced a destabilizing effect on the protein which increased its degradation, or an effect on the sorting process which had increased its retention in the endoplasmic reticulum. To test the possibility of the decrease in transporters, we measured Li^+ influx. ScTRK1 transports Li^+ exhibiting a K_m similar to that of Na^+ but a much lower rate. In the *trk1 trk2* mutant transformed with *ScTRK1*, Li^+ influx exhibited a kinetic made up of two components, one corresponded to the ectopic uptake of *trk1 trk2* mutants [4], which exhibited very low affinity, and the other corresponded to the uptake mediated by ScTRK1. Despite the much higher V_{max} of the ectopic transport, the second component could be isolated and studied by mathematical analysis of the data (Fig. 2 illustrates this fact showing an Eadie–Hofstee plot). Interestingly for our mutational study, the kinetics of Li^+ influx mediated by our mutants was not significantly different from the kinetics of the wild-type transporter shown in Fig. 3, which indicated that the mutations did not change the V_{max} of Li^+ influx. As a further support of this conclusion, we made a more extensive statistical analysis of Li^+ influx in several mutants at 2 and 5 mM Li^+. At these concentrations, the transport mediated by ScTRK1 made an important contribution to the total rate, and the capacity to detect a change in the rate mediated by ScTRK1 was maximal. If the large decreases in the Rb^+ V_{max}'s produced by the mutations had been the result of large decreases in the number of transporters, the V_{max}'s of Li^+ influx should have

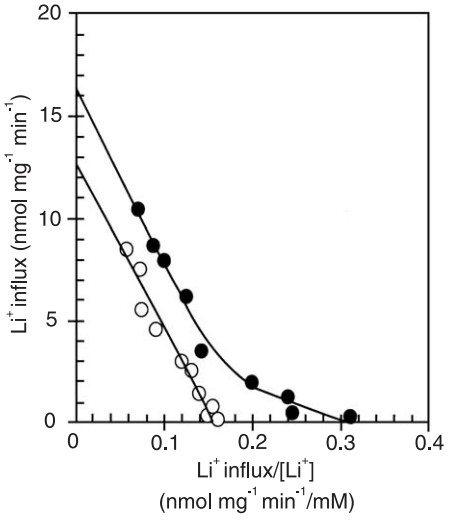

Fig. 3. Eadie–Hofstee plot of the ScTRK1 mediated Li^+ influx. The same plot for the recipient strain WΔ6 is also shown. The drawn lines are only for highlighting the main characteristics of the data points. When necessary, calculations have been carried out by nonlinear regression analysis.

Table 2
Initial rates of Li^+ uptake in wild-type ScTRK1 transporter and in K1147N, M1153R, and R1156S/K1158M/R1160I/N1159D mutants

Transporter	5 mM	10 mM
	nmol mg^{-1} min^{-1}	nmol mg^{-1} min^{-1}
Recipient strain, WΔ6	0.78 ± 0.22 (n=5)	1.41 ± 0.08 (n=4)
ScTRK1	1.37 ± 0.26 (n=7)	1.91 ± 0.19 (n=5)
K1147N	1.12 ± 0.13 (n=5)	2.20 ± 0.30 (n=5)
M1153R	1.20 ± 0.17 (n=8)	1.90 ± 0.27 (n=6)
R1156S/K1158M/ R1160I/N1159D	1.15 ± 0.06 (n=4)	1.85 ± 0.07 (n=4)

K^+-starved cells of the *trk1trk2* (WΔ6) yeast mutant expressing wild ScTRK1 transporter or its mutants were suspended in testing buffer and exposed to different Li^+ concentrations. The initial rates of Li^+ uptake were calculated from the time courses of Li^+ accumulation.

suffered proportional decreases, which did not occur. For example, in M1153R and quadruple mutants, the Rb^+ V_{max}'s decreased more than 10-fold, whereas the Li^+ V_{max}'s in these mutants were not significantly different from the Li^+ V_{max} of the wild-type transporter (Table 2) (it is worth observing that at the tested Li^+ concentrations, the kinetics of both the ectopic transport and the transport mediated by ScTRK1 approach to first-order kinetics exhibiting kinetic constant amounting to V_{max}/K_m). These results not only ruled out that the mutations decreased the number of transporters and implied that they decreased the V_{max} of Rb^+ influx but also that, despite their effect on Rb^+ transport, they did not affect Li^+ transport.

4. Discussion

Structural models of 4 × M1PM2, KcsA-related K^+ transporters locate the unique $M2_D$ helix in the internal part of the transporter, forming part of the pore and interacting with the innermost part of the P segments that forms the selectivity filter. This helix is tilted with reference to the axis of the pore and spans the cytoplasmic half of the membrane that is not spanned by the P segments. The polar characteristic and the relatively high number of positively charged residues in the $M2_D$ helix of eukaryotic transporters (Fig. 1B) is striking. It has been proposed that this helix stays buried in the membrane because it is very little exposed to the lipid alkyl chains, and has the positively charged residues interacting with negative residues in other fragments of the transporter [2], but nothing has been suggested about the functions of these positively charged residues.

Although some details require specific discussion, the first conclusions that can be drawn from our mutational study with the TRK transporter of *S. cerevisiae* supports the model in which the $M2_D$ helix forms part of the pore and interacts with P segments. Individual mutations removing a positively charged residue or adding a negatively charged one (K1147N, R1156S, K1158M, N1159D, and M1153E) produced significant changes on the Rb^+ K constants, possibly affecting the selectivity filter formed by the P segments, and on the Rb^+ V_{max}, possibly by affecting the structure of the pore. However, despite the variety of mutations, the changes were similar, except in mutation R1160I, which was without effect. These results are surprising because they failed to identify particularly deleterious mutations, which should have revealed crucial salt bridges between positive residues and negatively charged residues in P loops. It is a fact that a critical positively charged residue could not be identified. This suggests that specific salt bridges between particular positively charges residues in $M2_D$ helix and glutamate residues in P_B or P_D might not exist or are functionally irrelevant. Interestingly, the effects of the mutations on Rb^+ transport were additive on the V_{max} but not on the K_1. For example, mutations R1156S, K1158M, or N1159D increased the K_1's from 0.38 to 1.4, 0.94, and 0.97 mM, respectively, but the inclusion of the two first mutations and R1160I together increased the K_1 only up to 1.7 mM, and the further inclusion of N1159D did not have an appreciable effect. In contrast, the Rb^+ V_{max} was reduced to approximately a half by the single mutations and much more when three or four mutations were put together (Table 1).

Assuming that the $M2_D$ helix forms part of the pore, the generation of negative charges either directly by mutation or indirectly when the mutation eliminated a positive charge in the $M2_D$ helix were not extremely deleterious for the passage of Rb^+. According to the model, the generation of a positively charged residue in $M2_D$ should have a more dramatic effect on transport than the generation of negative charges, due to the formation of an electrostatic barrier that hardly could be penetrated by a cation. Consistent with this notion, the M1153R mutation decreased the Rb^+ V_{max} to almost undetectable levels, whereas mutations K1147N, R1156S, K1158M, R1160I, or N1159D produced milder effects (Table 1). However, all these explanations fail when the transport of Li^+ is considered, because unspecific effects of unpaired negative or positive charges should affect similarly to Rb^+ and Li^+ transport, which was not the case. In the quadruple R1156S/K1158M/R1160I/N1159D and single M1153R mutants, Rb^+ transport was almost completely abolished (Table 1) but Li^+ transport was not significantly changed (Table 2). All the results together suggest that the positive charges that exist in the ScTRK1 transporter are involved in driving the passage of Rb^+ through the pore, independently of whether they are paired or not to specific negative charges in other helixes. Thus, the removal of all positive charges or the addition of one in an inappropriate position affected the interactions of Rb^+ with the pore but probably not by producing electrostatic barriers, because in this case, the passage of Li^+ would have been also affected. A more likely explanation to the dramatically different effects of the mutations on the passage of Rb^+ or Li^+ is that the charge amino acid residues interact with Rb^+ but not with Li^+. This smaller cation could pass slowly through the pore as a secondary leak.

From the functional point of view of TRK1 transporters, the absence of an effect of mutation R1160I in this well-conserved positively charged residue poses a question about the selective pressure that conserves this residue (Fig. 1B). This question cannot be answered now and may be related to the multiple functions of TRK transporters that have not been investigated in this work. In *S. cerevisiae*, the TRK1 transporter has a variable K_m, depending on the nutritional status of the cells [1], and is involved in the regulation of the membrane potential and in other functions [5,6]. The Arg^{1160} residue may play an important role for these functions, or in others that have not been studied so far, and for this reason is conserved.

The TRK1 transporter of *S. cerevisiae* binds two Rb^+ cations for completing its transport cycle and it has been proposed that the two binding sites are independent but physically close in the selectivity filter formed by P segments [11]. The unconnected effects of the studied mutations on the K_1 and K_2 constants give further support to the existence of two physically independent sites for Rb^+ binding. For example, both K1147N and R1156S mutations increased 3.7-fold the K_1, whereas the K_2 increased 37-fold in the former and 10-fold in the latter. More interestingly, the M1153E mutation affected only the K_2, whereas another mutation in the same amino acid, M1153Q, affected only the K_1. The mild effects of the studied mutations on the Rb^+ K_1 correlate with their mild effects on the Na^+ K_i, which in addition were not related to the much more important changes produced on the K_2 in some of them. This is consistent with previous results obtained with mutants in the P_A fragment [11] and confirmed that Na^+ interacts only with one of the two cation binding sites of the transporter.

Special attention deserves mutation N1159D, which affected dramatically the K_2 and had medium effect on the K_1. Comparison of the Rb^+ influx kinetics of mutants Q90R (1.0 mM K_1 and 0.19 mM^2 K_2) [11] and N1159D (0.97 mM K_1 and non-detectable K_2) (Table 1 and Fig. 2) illustrates the two limits of the function of the TRK family of transporters. Mutant Q90R exhibits a notable sigmoid kinetics at concentrations below that producing half-maximal rate, compatible with a Rb^+–Rb^+ symport [11], whereas N1159D exhibits a Michaelis–Menten kinetics at all testable concentrations (Fig. 1), typical of a Rb^+ uniport. At the moment, the question of why single-amino-acid mutations produce such important functional changes in the transporter cannot be answered, nor can the important question of whether this important kinetic changes reflect real functional changes. The research in this direction is relevant in also its application to plant HKT transporters, because it is notable that wheat HKT1 behaves as a K^+–Na^+ symporter [22] and rice HKT1 behaves as a Na^+ uniporter (B. Garciadeblas, M.E. Senn, M.A. Bañuelos, and A. Rodríguez-Navarro, unpublished results). The understanding of the molecular basis of these differences is probably tight to the understanding of the differences between symporters and uniporters.

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