Change to alanine of one out of four selectivity filter glycines in KtrB causes a two orders of magnitude decrease in the affinities for both K^+ and Na^+ of the Na^+ dependent K^+ uptake system KtrAB from *Vibrio alginolyticus*

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Abstract KtrAB from *Vibrio alginolyticus* is a recently described new type of high affinity bacterial K^+ uptake system. Its activity assayed in an *Escherichia coli* K^+ uptake negative mutant depended on Na^+ ions (K_m of 40 μM). Subunit KtrB contains four putative P-loops. The selectivity filter from each P-loop contains a conserved glycine residue. Residue Gly-290 from the third P-loop selectivity filter in KtrB was exchanged for Ala, Ser or Asp. KtrB variants Ser-290 and Asp-290 were without activity. In contrast, KtrB variant Ala-290 was still active. This variant transported K^+ with a two orders of magnitude decrease in apparent affinity for both K^+ and Na^+ with little effect on V_{max}.

Key words: K^+ symporter family; Na^+/K^+ symporter; HKT1; P-loop; Selectivity filter; KcsA-K^+ channel family

1. Introduction

The recently determined structure of the K^+ channel KcsA from *Streptomyces lividans* shows how this channel folds in the membrane [1]. Each of the four identical subunits from the complex consists of an N-terminal outer transmembrane helix (M1), a P-loop region (P) and a C-terminal inner transmembrane helix (M2, Fig. 1A). An essential element of the P-loop is its helix, which enters the membrane from the outside with an angle of about 45° to the plane of the membrane and points towards the center of the channel (skewly boxed region in Fig. 1A). The P-loop helix ends at a depth of 15 Å inside the membrane with a turn followed by the selectivity filter residues GYG. The main chain of these residues is outwardly directed and has an extended conformation. It is connected by a turn to helix M2. The selectivity filter residues GYG of the four subunits together form the narrowest part of the channel. The main chain carbonyl groups from the filter region TVGY participate in the binding of dehydrated K^+ ions, forming two K^+ binding sites at each end of the narrow pore region. The side chains of the selectivity filter tyrosines point away from the channel center and interact with a tryptophan residue (W68 in KcsA, see Fig. 1A) from the P-loop helix. Together with the vicinal P-helix residue W67 these three aromatic residues form a hydrophobic cuff around the narrow pore in the selectivity filter region [1].

Jan and Jan [2] have proposed that the K^+ translocating subunit TrkG from one of the two *Escherichia coli* Trk K^+ uptake systems also contains two P-loop regions. Subsequently it was recognized that TrkG belongs to a broad family of K^+ symporter proteins [3–5], consisting of HKT1 from plants [6], Trk1 and Trk2 from fungi [7,8], KtrB (NtpJ) from bacteria [3,9], and TrkH (TrkG) from prokaryotes [10,11]. Independently, the Durell-Guy group and we recognized that these proteins consist of a basic structure of four consecutive M1-P-M2 motifs ([5,12], see Fig. 1B for KtrB from *Vibrio alginolyticus*). Alignment and modelling studies showed that each of these four segments contains a P-loop-helix like sequence in all four groups of proteins ([5,12], skewly boxed regions in Fig. 1B). However, the selectivity filter regions of the symporters are simpler than those of the K^+ channels and consist of only one glycine residue which is absolutely conserved in all four P-loops of all four groups of K^+ symporters ([5], white on black residues in Fig. 1B). These glycine residues occur at a position equivalent to the N-terminal glycine from the selectivity filter GYG residues of the K^+ channel proteins [12]. It is assumed the the family of K^+ symporters contain only one K^+ binding pocket in their selectivity filter region [12].

KtrB is the K^+ translocating subunit of the recently described new type of bacterial K^+ uptake system KtrAB from *V. alginolyticus* [3]. It had previously been described as NtpJ from *Enterococcus hirae* [9,13] and occurs in many other bacteria [3,14]. Of the four groups of K^+ symporters KtrB resembles KcsA most closely [5]. Since *V. alginolyticus* KtrAB activity is easily assayed in *E. coli* [3], KtrB is the ideal protein for mutagenesis studies with the aim of testing the hypothesis that this family of proteins is composed of four consecutive M1-P-M2 motifs. Here we report that K^+ uptake via KtrAB is Na^+ dependent and that the change of the selectivity filter glycine to alanine in the third putative P-loop from KtrB causes a two orders of magnitude decrease in affinity for both K^+ and Na^+ of K^+ uptake via the KtrAB system.

2. Materials and methods

2.1. Strains and plasmids

Strain LB2003 F^- *kup1* Δ*kdpABC5* Δ*trkA rpsL metE thi rha gal* [15] and plasmid pKT84, containing the *ktrAB* genes from *V. alginolyticus* [3] have been described previously. Mutations in codon 290 of *ktrB*

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Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid

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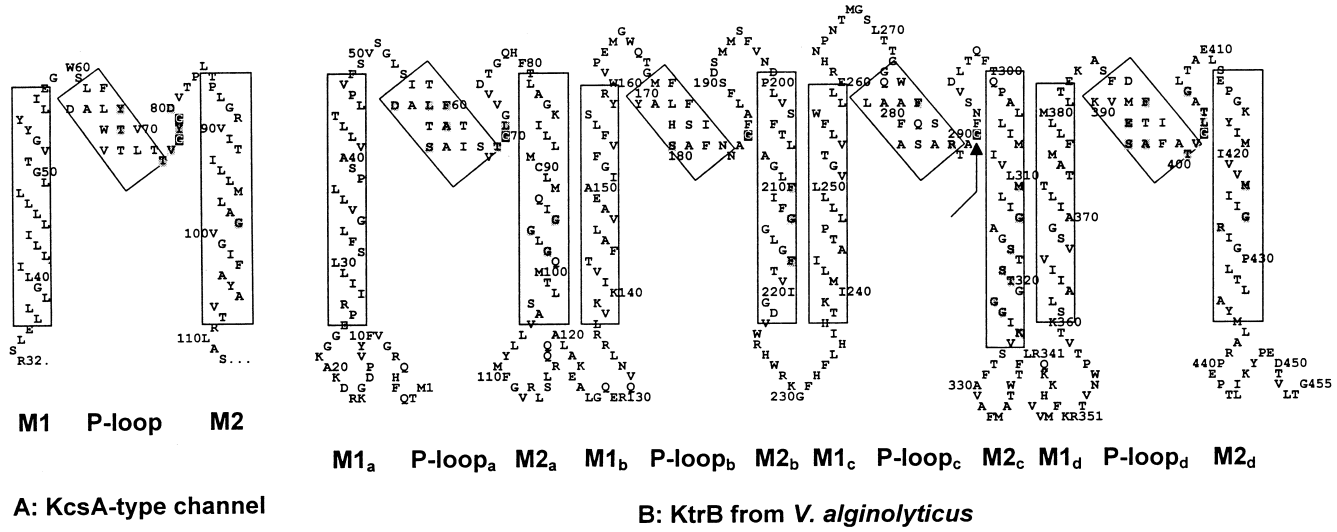

Fig. 1. Schematic drawing of (A) the KcsA type of prokaryotic K^+ channel and (B) subunit KtrB from the K^+ uptake system KtrAB from *V. alginolyticus*. A: Consensus sequence deduced from 28 KcsA type sequences from prokaryotes [5]. Universally conserved residues are given in white on a black background; often conserved residues are printed bold on a gray background. Membrane helices M1 and M2 and the P-loop helix [1] are boxed. B: Representation of KtrB according to the model of [5,12]. The four repeating elements M1, P-loop, M2 are indicated by the letters a–d. Universally conserved and often conserved residues in KtrB, based on 18 bacterial KtrB sequences [5], helices M1 and M2, and P-loop helices are highlighted as in A. Residue glycine 290 is marked with an arrow. A sequencing error in *V. alginolyticus ktrB* has been corrected here: codon 186 translates as glycine (B) instead of alanine [3].

were introduced with the overlap extension method using the polymerase chain reaction [16]. With this method the glycine codon GGT was replaced by GCT (alanine), GAT (aspartate), and AGT (serine). Subsequently, a 0.25 kb *Nco*I-*Dra*III fragment containing the mutation was exchanged for the same, but wild type fragment from plasmid pKT84 [3], giving rise to plasmids pNT21, pNT22 and pNT23, respectively. It was checked by nucleotide sequencing that the fragments inserted in pKT84 only contained the desired mutation.

2.2. Growth conditions, K^+ depletion and K^+ uptake assay

Cells of strain LB2003 containing plasmids pKT84, pNT21, pNT22, or pNT23 were grown at 37°C under aerobic conditions in minimal mineral medium K30 containing 12 mM K_2HPO_4, 6 mM KH_2PO_4, 34 mM Na_2HPO_4, 17 mM NaH_2PO_4, 8 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, 0.6 μM $FeSO_4$, 1 mg/l thiamine, 40 mg/l methionine, 100 mg/l carbenicillin, and 10 mM glucose [17].

Cells were harvested during the late exponential phase, depleted of most of their K^+ ions by Tris-EDTA treatment [18], washed three times with and resuspended in 200 mM HEPES buffer brought to pH 7.5 with either NaOH or triethanolamine hydroxide [19], and shaken at 20°C before being used in the K^+ uptake assay. For this assay cells were suspended at 1 g dry wt/l (corresponding to an optical density of 3 at 578 nm) in the 200 mM sodium HEPES or triethanolamine-HEPES buffer at 37°C. The suspension was shaken at 180 rpm. 10 mM glucose was added at $t = -10$ min. At $t = 0$ KCl was added at the concentration indicated in the figure legends. NaCl was added at either $t = 0$ (together with KCl) or at $t = 6$ min to the suspension in the experiment of Fig. 2A. Samples of 1.0 ml were centrifuged through silicone oil (AR200, Serva, Heidelberg, Germany) at different times. The K^+ contents of the cell pellets were measured by flame photometry with an ELEX 6361 instrument (Eppendorf, Hamburg, Germany). Na^+ contents of the cell suspension were determined with the same technique.

3. Results

3.1. KtrAB activity is sodium dependent

K^+ uptake via the plant HKT1 system is sodium dependent [20]. Since HKT1 and KtrB are members of the same protein family, we tested whether KtrAB activity is also sodium dependent. For this purpose we used cells of the K^+ uptake negative *E. coli* strain LB2003 containing the *V. alginolyticus ktrAB* genes on plasmid pKT84 [3]. We have previously shown that K^+ depleted cells of this strain rapidly accumulate K^+ from the medium when suspended in buffer containing 10 mM glucose and sodium HEPES, pH 7.5 [3]. Under similar conditions cells washed with and suspended in the triethanolamine-HEPES buffer took up hardly any K^+ until Na^+ was also added (Fig. 2A). The half-maximal effect for Na^+ stimulation of K^+ uptake occurred at about 40 μM (results not shown). As a control we checked the activity of the *E. coli* Trk systems in strain LB2003(pSS420) [15]. We did not observe a significant dependence on Na^+ ions of K^+ uptake by this strain (Fig. 2B), confirming that K^+ uptake via the *E. coli* Trk systems is sodium independent [19]. The same was true for the Trk system from *V. alginolyticus* (results not shown).

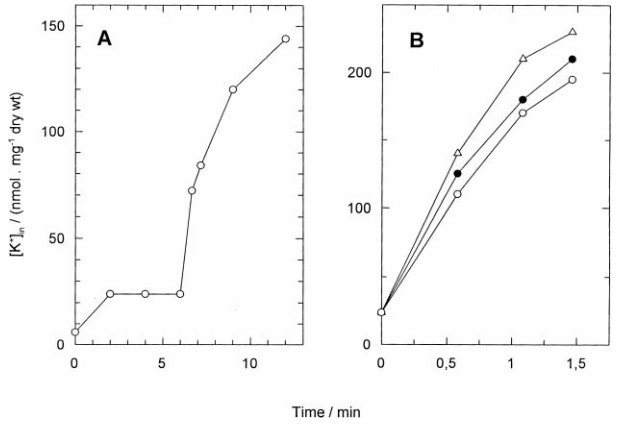

Fig. 2. K^+ uptake activity of KtrAB depends on Na^+ ions. EDTA treated cells of *E. coli* LB2003 (pKT84) (A) or LB2003 (pSS420) (B) were washed with and suspended in 200 mM triethanolamine-HEPES buffer, pH 7.5. In A, 10 mM glucose, 500 μM KCl and 500 μM NaCl were added at $t = -10$, 0, and 6 min, respectively. In B, 10 mM glucose was added at $t = -10$ min, 2 mM KCl was added at $t = 0$ together with 0 (●), 25 (○) or 50 μM NaCl (△).

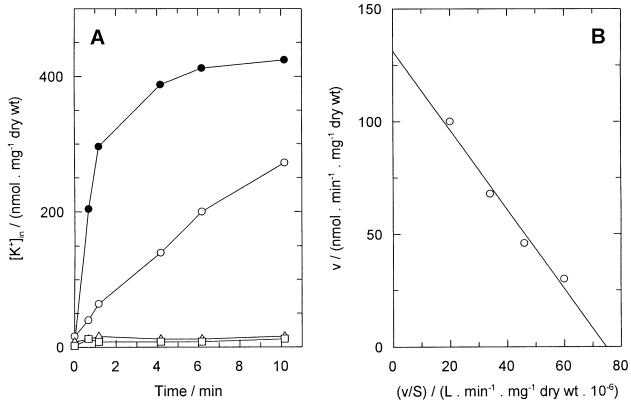

Fig. 3. K^+ uptake by KtrB residue-290 variants. Uptake was assayed in the sodium-HEPES medium. In A, KCl was added at $t = 0$. Symbols: ●, wild type (KtrB residue Gly-290); ○, variant Ala-290; □, variant Ser-290; △, variant Asp-290. B: Eadie-Hofstee plot for initial rates of K^+ uptake by the Ala-290 variant.

3.2. KtrB variants at position 290

The conserved residue Gly-290 from the third P-loop in KtrB (Fig. 1B) was changed to alanine, serine or aspartate. Only the change to alanine was tolerated. At $[K^+]_{out} = 500$ μM cells containing this KtrB variant transported K^+ much more slowly than did wild type cells. Cells with the KtrB variants Ser-290 or Asp-290 were devoid of K^+ uptake activity (Fig. 3A). By varying the K^+ concentration in the medium we established that the K_m value for K^+ uptake changed from below 50 μM in the wild type [3] to about 1.7 mM in the KtrB Ala-290 variant (Fig. 3B). Compared to this large change, the effect on V_{max} was minor (about 200 and 130 nmol min^{-1} mg^{-1} dry wt. for the wild type and the variant, respectively ([3] and Fig. 3B, respectively). A similar effect was observed for $^{86}Rb^+$ uptake by KtrAB, for which the K_m value changed from about 1 mM for the wild type [3] to about 30 mM for the variant and V_{max} of the variant was lower by a factor of 4 (results not shown).

3.3. Na^+ dependence of K^+ uptake by the Ala-290 variant

The effect of Na^+ on K^+ uptake via the Ala-290 KtrB variant was tested. Remarkably, the K_m value of K^+ uptake for Na^+ also increased by two orders of magnitude to about 6 mM for the variant (Fig. 4).

4. Discussion

Although Na^+ linked K^+ uptake systems have been predicted to exist in prokaryotes for some time [20], KtrAB is the first real example of such a system. K^+ uptake via KtrAB provides a further example of a Na^+ linked membrane function in *V. alginolyticus*. For this bacterium it has been shown that the processes of electron transport from NADH to coenzyme Q [21], flagellar rotation [22], and solute uptake [23] require Na^+ ions. The *ktrB* (*ntpJ*) gene of the bacterium *E. hirae* is located at the 3′ end of a cluster of Na^+ transport genes [10]. Hence, we expect that the KtrAB system from this organism is also Na^+ dependent, and it may well be that all bacterial KtrAB systems are sodium dependent.

The cation with which K^+ is cotransported via proteins of the HKT1-Trk1,2-KtrB-TrkH family varies for the different subgroups of this family. K^+ uptake via both HKT1 from wheat roots and KtrAB from *V. alginolyticus* depends on Na^+ ions ([24] and this work, respectively). In contrast, at least one fungal Trk system [25] and the *E. coli* Trk systems [26] have been reported to mediate H^+ linked K^+ uptake. In this work we reinvestigated the effect of Na^+ on the latter as well as on Trk from *V. alginolyticus*. Full K^+ uptake activity was observed in the absence of added Na^+ ions at a contaminating $[Na^+]$ concentration in the medium of about 5 μM (Fig. 2B and results not shown). Hence, we conclude tentatively that these systems do not require Na^+ for activity. However, the possibility remains that these Trk systems have a very high affinity for Na^+ (K_m for $Na^+ \leq 1$ μM).

The affinity of the KtrAB system system for Na^+ (K_m-value of about 40 μM) is similar to that of the Na^+/proline symporter PutP from *E. coli* [27]. For KtrAB it remains to be established whether the observed stimulation of K^+ transport activity by Na^+ arises from Na^+/K^+ symport or from binding of Na^+ to a K^+ uptake activating site. Since HKT1 is a Na^+/K^+ symporter [4,24], we expect KtrAB to function similarly.

In a shaker type of K^+ channel the change of the N-terminal selectivity filter glycine to cysteine in one out of four subunits is not tolerated [28]. Here we report on a similar effect. When one out of the four putative selectivity filter glycines from the K^+ transporting subunit KtrB is changed to either serine or aspartate, KtrAB loses its activity (Fig. 3A). However, the change to the smaller alanine residue at this position is still tolerated (Figs. 3 and 4). This change leads to a dramatic decrease in the affinities of both Na^+ and K^+ for K^+ uptake (Figs. 3B and 4B). A similar effect has been described for the Na^+/proline symporter PutP, in which the change of residue serine at position 57 to alanine, cysteine, glycine or threonine caused a two orders of magnitude decrease in apparent affinity for both Na^+ and proline with little change in V_{max} [29,30]. For KtrAB this type of effect can be explained in three ways: (i) K^+ and Na^+ move through the same pathway, i.e. through the pore formed by the selectivity filter glycine residues; for symporters this appears to be an unlikely mechanism [12] for which one has to assume that K^+ and Na^+ file through the pore in alternating order; (ii) in these proteins there exist overlapping Na^+ and K^+ binding sites in which the changed amino acid residue participates.

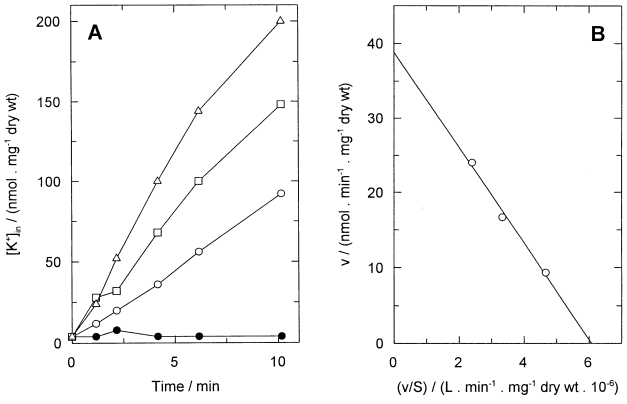

Fig. 4. Two orders of magnitude decrease of Na^+ affinity for K^+ uptake via the KtrB Ala-290 variant. A: Cells were assayed in the triethanolamine-HEPES medium. 2 mM KCl and NaCl were added at $t = -5$ min and $t = 0$, respectively. Symbols: ●, control (no NaCl added); ○, □, and △, 2, 5, and 10 mM NaCl, respectively. B: Eadie-Hofstee plot for the initial rates of K^+ uptake from A.

Such a model has been proposed for the effects of exchange of residue serine 57 in PutP [30]; and (iii) the binding of one substrate to the protein (K^+ for the KtrB-A290 variant) causes a conformational change in the protein, leading to a decreased affinity of the protein for the second ion (Na^+ in KtrB). The structural model for KtrB predicts that glycine residue 70 from P-loop a in KtrB is located at the opposite side of the K^+ pore compared to residue Gly-290 ([5,12], Fig. 1B). According to mechanisms ii or iii one predicts that the glycine to alanine exchange will cause smaller effects on the affinity of the system for Na^+ than for K^+. Experiments are under way to test this hypothesis. Diatloff et al. have already shown that two changes in the putative P-loop helix d from HKT1 lead to a decreased affinity of the system for Na^+ only [4].

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