

MjK2, a K⁺Channel from *M. Jannaschii* Mediates pH Dependent Potassium Transport Activity

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Abstract

MjK2 was expressed in *E. coli* cells as a fusion protein containing N- or C-terminal an antibody binding site and a histidinehexamer. The C-terminal tagged fusion protein allows the expression and purification of an extra soluble RCK domain at p34 kDa, whereas this additional RCK domain was lost when the N-terminal tagged construct was used. Upon removal of the fusion peptide from the purified N-terminal tagged channel monomer, MjK2 occurred as a stable tetramer when incubated with synthetic lipid. The channel activity was studied after reconstitution into liposomes by single channel recording or by an optical assay with the potassium sensing dye, PBFI. First the channel function was improved by single channel recording. Single channel recording confirmed the pH dependence of the channel activity with single channel conductances of 42, 70, 85 and 202 pS and indicated that a functional K⁺ channel was formed. To study the function of the reconstituted MjK2 activity in an optical assay the potassium release was initiated when the external BaCl₂ block was compensated by addition of EDTA. The release of potassium was mediated by reconstituted MjK2 at low pH or by the presence of internal calcium at high pH. MgCl₂ had no or weak effect, whereas cAMP at low pH caused a complete loss of potassium during the preparation. Alignments studies revealed that MjK2 has different structural features in the channel pore and the RCK composition and therefore a different function can be expected. Amino acid sequence and structural alignments showed that a Ca²⁺ binding site and a typical nucleotide-binding site is not present in the RCK domain of MjK2 and therefore a different behavior could be expected. In addition a lysine reach linker region as found in human sperm K⁺ channels hsl01 and hsl03 can play similar role in the gating behavior.

Keywords: MjK2; RCK; Reconstitution; Single channel recording; PBFI

Abbreviations: RCK: Regulator of Conductance of Potassium; MthK: *M. thermoautotrophicum*; MjK2: K⁺ channel 2 of *M. jannaschii*; POPC: Phosphatidylcholine; POPG: Phosphatidylglycerol; PBFI: Benzofuranisophthalate dye

Introduction

The K⁺ channel MjK2 from *Methanococcus jannaschii* is an MthK homologue [1]. Crystallographic studies of the MthK channel together with functional studies reveal an allosteric mechanism of Ca²⁺-binding and activation, while increased H⁺ inhibited MthK gating [2-6]. The MthK channel structure is organized with four protomers that build the pore and with four extra soluble RCK domains (regulator of K⁺). Together with these four additional cytoplasmic separate RCK domains the channel form an octameric gating ring with 24 Ca²⁺-binding sites [7,8]. The RCK domains are known to control the activity of a variety of K⁺ transporters and channels [9-12]. Divalent cations and pH regulate the activity of these channels, as shown for the MthK channel and others, but several other RCK domains with conserved folding patterns known as KTN (K⁺ transport, nucleotide binding) domains have a classical nicotinamide dinucleotide-binding motif. Since the cytoplasmic MthK domain fits to that of BK channels it is suggested that the BK channels could provide a model for Ca²⁺ activated K⁺ channels without typical voltage sensor domains [2,13,14]. Human BK channels (BK; Big Potassium), also called Maxi-K or slo1 which are important pharmacological targets for the treatment of several medical disorders including stroke and overactive bladder.

Two RCK K⁺ channel genes identified in the *Methanococcus jannaschii* genome, MjK1 and MjK2 share high similarity, but as shown before the two RCK channels had different behavior in complementation studies [1]. In addition, MjK1 forms stable channel oligomers after SDS-PAGE separation. In complementation assays in *E. coli* strain LB2003, MjK1 mediated K⁺ uptake for sub-toxic K⁺

concentrations. MjK2 is unstable and does not mediate K⁺ uptake at 1 mM [1]. In addition, purified MjK2 exhibited Ba²⁺-sensitive ⁸⁶Rb⁺ transport activity after mild solubilization, which promotes the formation of stable channel oligomers (SEC and SDS-PAGE) [15]. Interestingly, the RCK domain of MjK2 alone binds to negatively charged lipids, which indicates an additional or different function of MjK2. As shown in KcsA folding studies, anionic lipids are essential for the function and stability of the membrane portion of the channel [16]. These data have also shown that MjK2 does not behave similarly to MthK during assays or purification.

Since it was shown before by alignment studies, MjK2 has like other K⁺ channels a RCK domain which is not related to the Ca²⁺ or nucleotide-binding site of other RCK channels or transporters a functional characterization was performed [1,17,18]. To study the folding and stability of MjK2 channel tetramers, the protein was over-expressed in *E. coli* cells and purified by affinity columns. Additionally, the conditions for channel activity of the reconstituted MjK2 channelosomes studied on single channel level or screened by an optical assay with benzofuran isophthalate dye (PBFI) to detect potassium transport after compensation of the Ba²⁺-blockade by EDTA. PBFI as fluorescent K⁺ indicator [19] has been widely used in

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several applications to examine K⁺-selective ion transport, modulation [20] or for inhibitor screenings [21].

Material and Methods

Material

POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)) were obtained from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). Cobalt-chelating resin (TALON) purchased from Takara, (Saint-Germain-en-Laye, France); OG (n-octyl β-D-glucopyranoside) from Glycon Biochemicals GmbH, (Luckenwalde, Germany) and Topo cloning kits from Life Technologies, GmbH, (Darmstadt, Germany).

Bacterial strains

The *E. coli* strain TOP10 and BL21plys (Invitrogen) was used to host the plasmids pGEMHE and pTrcHisTopo or pEXP5Topo containing the hCx26 gene, respectively.

Cloning of MjK2

MjK2 with a C-terminal fusion site was obtained as previously described [1]. To generate N-terminal tagged MjK2, the open reading frame of Mj1357 gene was ligated into pTrcHisTOPO (Invitrogen) (fwd: 5'-ATG GAG ACG TCA AAG AA-3', rev: 5'-AGCCAAGCTTTCATAACTTTTCTT-3). The resulting plasmid, pMjK2Nt, provides an N-terminal histidinehexamer sequence, an X-press epitope and cleavage sites for enterokinase.

Protein purification

For protein expression MjK2 containing plasmids were transferred into *E. coli* BL21plys cells and from an overnight culture a sample was transferred a ratio of 1:200 into a bioreactor (1.5l) containing terrific broth and 5% glycerine and induced with 1.1 mM IPTG at an OD₆₀₀ of around 16 at 37°C for 6.5h. *E. coli* membranes were prepared as described previously [22]. The conditions for the expression, detection and purification (using a detergent extract) of MjK2Nt, which was isolated from homogenized *E. coli* (BL21plys) cells, were described according to [20]. The homogenate was solubilized in 200 mL containing 1% N-lauroylsarcosine, 100 mM NaCl, 2 mM imidazole and 50 mM Tris-HCl (pH 8.0) and stirred on ice for 1h. The detergent extract was centrifuged at 10,000xg for 30 min at 4°C. The detergent concentration of the supernatant was reduced to 0.4% and incubated on ice overnight with 6 mL of cobalt-chelating affinity resin (Talon, Clontech). Subsequently, the resin was added to a column and washed with 30 mL containing 0.4% N-lauroylsarcosine, 150 mM NaCl, 2mM imidazole and 10 mM Tris-HCl, pH 7.0 to remove unbound proteins. MjK2Nt was eluted into a buffer containing 30 mM octylglucoside, 300 mM imidazole, 150 mM NaCl and 10 mM Tris-HCl at pH 7.0, filtrated on a 0.2 μm filter to remove precipitates and concentrated on Amicon YM30 filters. The concentrate was diluted three times 10 fold with 30 mM octylglucoside, 150 mM NaCl and 10 mM Tris-HCl, pH 8.0 to reduce the imidazole concentration. In a similar manner, was previously shown to purify functional KcsA [21]. The optical density of the purified MjK2Nt channel was estimated at 280 nm and the protein concentration was calculated using an extinction coefficient of 23,520 M⁻¹cm⁻¹ [23]. To remove the N-terminal fusion site (MGGSHHHHHHGMASMTGGQQMGRDLYDDDDDK; calc. mass of 3.4 kDa), the purified MjK2Nt channel (10 mg) was equilibrated with 1x enterokinase buffer and incubated with 0.005% (w/w) light chain enterokinase (NEB) for 24 h at RT. The digests were controlled using

immune blots, the fully digested MjK2 gave a band at 38.9 kDa. For folding experiments, lipid was added in a 1:1:0.1 (w/w; 1.3 mM/25.7 μM, 23.2 μM POPC, 2.5 μM POPG) lipid-to-protein ratio.

Thermostability assay

After the incubation of MjK2 oligomers in 30 mM OG, 150 mM NaCl and 10 mM Tris-HCl at pH 8 and the indicated temperature in a water bath for 15 min, the samples were transferred on ice and adjusted with SDS sample buffer (2% SDS, 2% β-mercaptoethanol, 0.1% bromphenol blue, 10% glycerol and 50 mM Tris-HCl, pH 6.8) to analyze the oligomer stability using SDS-PAGE.

Preparation of channelosomes and optical measurements

Channelosomes were prepared as described previously [22]. The POPC/POPG (10:1) lipid film was resuspended in solutions as indicated (Table 1, Figure 3). After sonication, the emulsions were incubated with purified MjK2 at a 100:1 ratio (10 mg mL⁻¹lipid (13.16 mM), 100 μg of MjK2 (2.6 μM) on ice for 1 h. Controls were prepared without MjK2, but with detergent buffer. The solution was diluted 5 times with cold buffer containing 1 M glycine, 10 mM BaCl₂, and 10 mM Tris-HCl at pH 8 and centrifuged for 15 min at 65,000 rpm (Beckman rotor 10.4) at 4°C. The pellet was resuspended in dilution buffer and centrifuged again; these final steps were repeated once again. Potassium transport mediated by MjK2 activity was detected optically with 10 μM benzofuranisophthalate dye (PBFI) in black 384 multi-well plates. The fluorescence emitted at 355 nm was measured in a Berthold multiplate reader. The potassium was monitored using the fluorescence intensity at 460 nm after the sample volume was adjusted with 10 μL of 50 mM EDTA to a final volume of 50 μL with a short injection after 5 s at 20°C as used previously [22].

Planar lipid bilayer measurements

Lipid bilayers were formed from a solution of 2% L-α-diphtanoyllecithin on a 200 μm hole in a Teflon partition that separated two 5 mL compartments (cis and trans), as used previously [22]. A volume of 10 μL of reconstituted channel was added to the cis compartment and fused into the lipid bilayer. The channel activity was measured, if not otherwise stated, in symmetrical K⁺-buffer (500 mM KCl and 20 mM K-gluconate, pH 4.0). The currents were measured using a low-noise current-to-voltage converter, filtered at 1 kHz with an eight-pole low-pass Bessel filter. The data were digitized using a 12-bit multifunction I/O board (PCI-MIO-16E-4, National Lab) and a 333 MHz Pentium II computer. The data acquisition and single channel analysis were performed with the LabVIEW, Vers. 5.0 (National Lab) and Origin Vers.5.0 (Northampton, MA) software packages.

Results

Purification of MjK2 and formation of stable channel tetramers

All three MjK⁺ channels (MVP, MjK1 and MjK2) could be expressed in *E. coli* [1,15]. Whereas MVP and MjK1 formed stable tetramers after separation using SDS gel electrophoresis, only monomers of the MjK2 subunit were obtained under identical expression and electrophoresis conditions [1]. To study the formation of stable oligomers of MjK2, the entire open reading frame was cloned into a bacterial expression vector and expressed in *E. coli* with a fusion site at the N-terminus. The protein was purified in a single step on a cobalt-chelating affinity column. The N-terminal tagged channel protein was measured with an apparent molecular mass of 43-kDa (Figure 1A, lanes 2 and 3),

Internal composition	10 Mm BaCl ₂ (external)	Intensity (355/460)	
		mean (n=2)	±s.d.
MjK2, 1 M KCl, pH ₄	+	9192.15	2124.87
MjK2, 0.1 M KCl, pH ₄	+	7284.97	2684.83
MjK2, 0.01 M KCl, pH ₄	+	5580.07	187.98
MjK2, 1 M KCl, pH ₈	+	5434.06	1441.39
MjK2, 0.1 M KCl, pH ₈	+	4949.42	1277.84
MjK2, 0.01 M KCl, pH ₈	+	4125.7	2071.16
MjK2, 1 M KCl, 25 mM MgCl ₂ , pH ₄	+	1970.32	477.66
MjK2, 1 M KCl, 25 mM MgCl ₂ , pH ₈	+	3854.24	1593.28
MjK2, 1 M KCl, 25 mM CaCl ₂ , pH ₄	+	3646.67	938.47
MjK2, 1 M KCl, 25 mM CaCl ₂ , pH ₈	+	8831.86	708.26
MjK2, 1 M KCl, 0.1 mM cAMP, pH ₄	-	-14885.43	1302.8
MjK2, 1 M KCl, 0.1 mM cAMP, pH ₈	+	4683.14	919.53
MjK2, 1 M KCl, pH ₄	-	-18902.9	3328.44
Liposomes, 1 M KCl, pH ₄	+	-1977.27	854.84

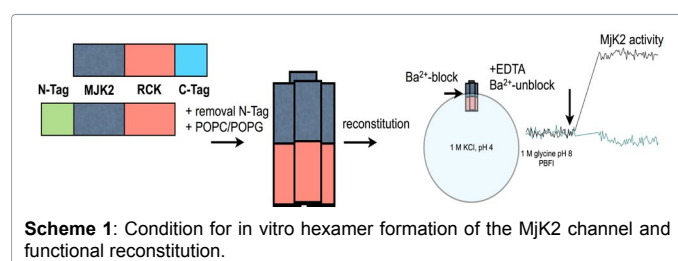
Table 1: Effect of the internal composition of channelosomes on the MjK2 activity. The optical activity of MjK2 or control liposomes was monitored as described in Figure 4.

which corresponds to the predicted size of the single channel subunit. In comparison, a prominent additional peptide, p34, was co-purified when a fusion site of 4 kDa at the C-terminus was used (Figure 1A, lane 1). From other RCK channels it is known that this 34 kDa assembles with the p43 to an oligomeric RCK channel. In contrast, p43 was eluted as a monomeric protein. The position of the tag (N- or C-terminal) does not influence the oligomerization behavior. The additional p34 protein was absent in N-terminal fractions as observed in C-terminal tagged MjK2 channel fractions. However, the reason for this difference was unknown, but it may indicate that the position of the tag influence also the p34 expression. On the other hand p43 and p34 does not form stable oligomeric complexes.

To elucidate whether the fusion site hinders the channel assembly, MjK2Nt was incubated with enterokinase to remove the N-terminal fusion site. As shown in Figure 1B, the addition of divalent cations, such as Mg²⁺, Ba²⁺ and Ca²⁺, or low pH (4.0) was able to increase the formation of stable dimers, which can be identified on SDS-PAGE at 78 kDa, to the same extent as the presence of ATP did, but a transition into channel tetramers did not occur (Figure 1B, lanes 1-4). Strong binding of IMAC-purified MjK2 on a cAMP nucleotide-binding resin was also observed. However, it was not possible to elute the protein into high ATP concentrations (up to 100 mM), but elution was possible in 1 M NaCl (data not shown), which may result from an interaction with the KTN motif. To determine whether the presence of lipid was able to induce folding and assembly into tetramers, purified MjK2 subunits without the N-terminal fusion site were incubated with a POPC/POPG mixture (Figure 1B, lane 5). With POPC/POPG, tetramers and dimers were visible. The thermal stability of the separated tetramers was analyzed using SDS-PAGE (Figure 2A). A sigmoidal curve with a midpoint at ~92°C described the tetramer-to-monomer transition (Figure 2B), which indicates the high stability of the tetrameric MjK2 channel. The result is summarized in Scheme 1.

Low pH activates MjK2, while an extra RCK domain is not essential for the channel function

To study the single channel function after reconstitution into bilayer membranes, MjK2 liposomes were fused to a planar lipid bilayer and the activity of single channels were recorded. Single channel fluctuations were observed when the KCl concentration was adjusted to 500 mM or higher and the pH of the solution was kept at pH 3.5-4. Figure 3A shows the single channel behavior of MjK2. The



Scheme 1: Condition for in vitro hexamer formation of the MjK2 channel and functional reconstitution.

recordings were obtained at an applied voltage of -100 mV, and single-channel conductances of 202, 85, 70 and 42 pS were observed (Figure 3A). Measurements of the single channel behavior of MjK2 at various pH values revealed that the open probability decreased above pH 5 with half-maximal activation at pH 4.3 (Figure 3B). The addition of CaCl₂ did not increase the channel activity or open probability at pH 7 (data not shown).

Additional divalent cations and cAMP influence the MjK2 activity

An optical K⁺ release assay was used to monitor different regulatory conditions for the refolded MjK2 channel, with liposomes of various internal compositions. This assay has been used before for inhibitor screening on the reconstituted KcsA channel [23]. The potassium release from channelosomes was monitored using the potassium-sensitive dye PBFI, whose fluorescence intensity increases with the potassium concentration [21]. The blockade of MjK2 activity by high external BaCl₂ (10 mM) was compensated by a rapid injection of high levels of EDTA (50 mM, Figure 4A, Arrow), since BaCl₂ was previously shown to be an inhibitor of MjK2 activity and was required to avoid a leakage of potassium from liposomes during the channelosome preparation [15]. With the injection of EDTA, only MjK2-liposomes mediate potassium release monitored as an increase of the fluorescence that culminates within 2-2.5 s at a plateau (Figure 4A, Trace 1), indicating a compensation of the blockade by external BaCl₂. Liposomes without the reconstituted MjK2 channel caused no fluorescence increase (Figure 4A, Trace 2). A second injection of EDTA caused a signal decrease, perhaps because of the dilution (Figure 4A; Trace 1, 2). A drastic signal decrease was also observed when MjK2 liposomes were prepared without external BaCl₂ (Table 1). To determine different conditions which influence MjK2 channel activity, the liposomes were charged with various KCl concentrations

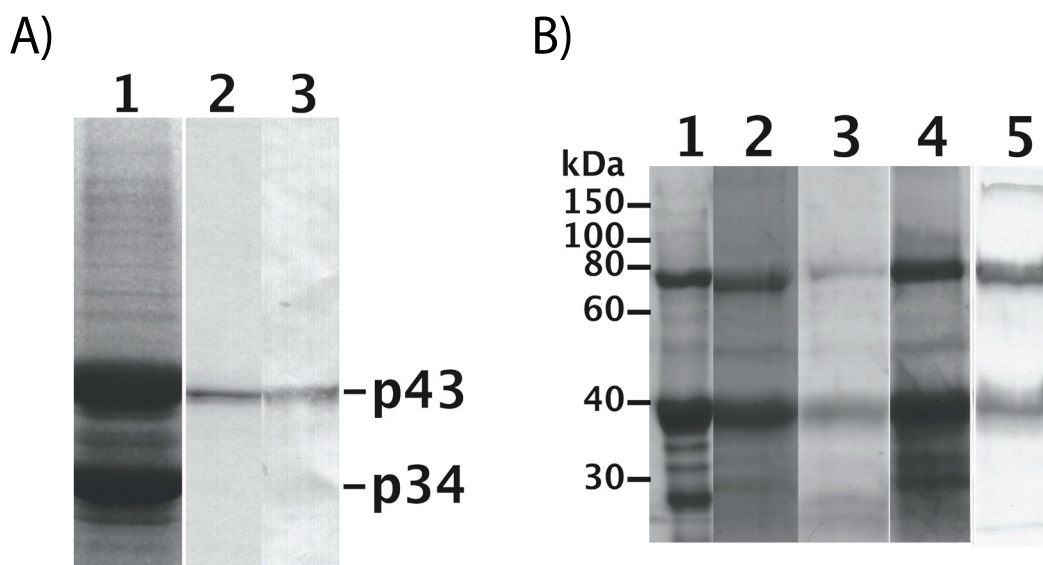


Figure 1: Single-step affinity purification of MjK2 constructs. MjK2 with elongated fusion sites at the N- or C-terminus were bound to a Co^{2+} -chelating column, washed with Na buffer (10 mM Tris-HCl, and 500 mM NaCl, pH 8,0) and eluted in a Na buffer supplemented with 300 mM imidazole. The purified proteins were stained with Coomassie blue (CO) or detected with an anti-X-press antibody (XP). a) (lane 1) C-terminal tagged MjK2CT (CO), (lane 2) N-terminal tagged MjK2NT (CO), and (lane 3) N-terminal tagged MjK2NT (XP) b) Effect of putative stabilizers on oligomer formation (CO). Incubation of purified untagged MjK2 in 100 mM KCl, 30 mM octyl glucoside, and 10 mM Tris at pH 8 (if not otherwise stated); (lane 1) 10 mM Ba^{2+} ; (lane 2) 10 mM Ca^{2+} ; (lane 3) 50 mM Mg-ATP; (lane 4) 10 mM K-gluconate pH 4.0; and (lane 5) POPG.

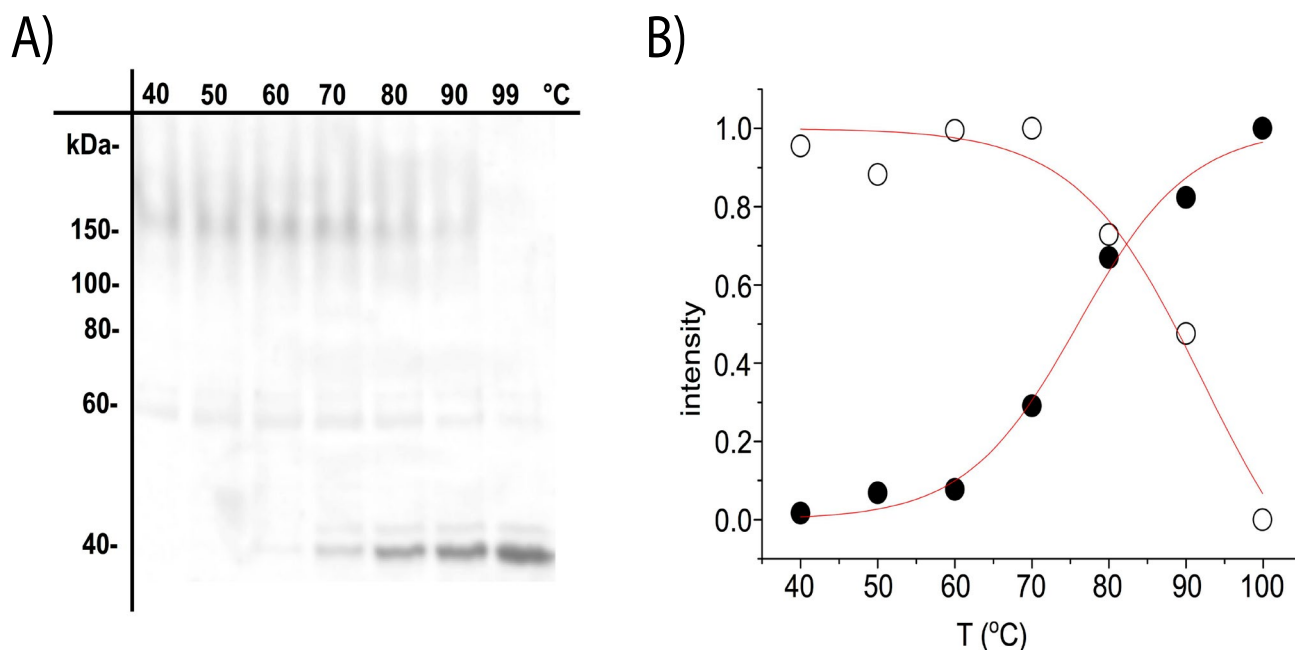
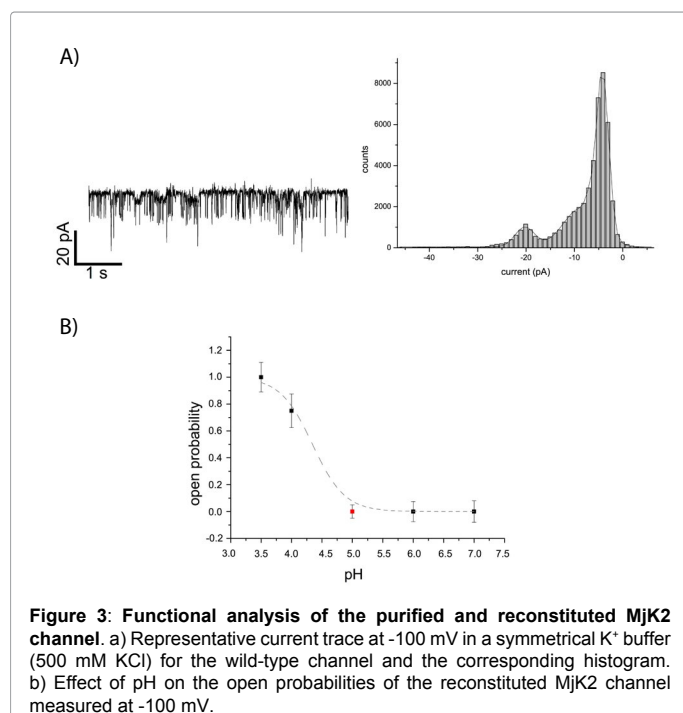


Figure 2. *In vitro* assembly of MjK2 and temperature-dependent dissipation. A) Coomassie-stained (CO) 10% SDS-PAGE gel of the thermal denaturation experiment of the MjK2 channel protein obtained after enterokinase treatment at different temperatures (40-99.9°C) and intensity plots (B) quantified by densitometric scans of the corresponding gel lanes. Open circles describe the oligomer-to-monomer transition, which can be fitted by a sigmoid curve with a mid-point at ~92°C. Closed circles represent the monomer-to-tetramer transition with a midpoint at ~83°C.



(0.01, 0.1, and 1 M) at pH 3.5 and 8 or in the presence of 1 M KCl and 25 mM MgCl₂ or 25 mM CaCl₂ or 0.1 mM cAMP, respectively (Figures 4B, C). To remove external potassium, the liposomes have been washed three times before transferred into the microplate, thus background fluorescence was around 7-9×10⁴ counts. The highest release activity occurs with liposomes loaded with 1 M KCl at pH 3.5 or when internal 1M KCl, 25 mM CaCl₂ and pH 8 used (Figure 4C, Table 1). The data also reveal that potassium release or the fluorescence intensity decreased in a potassium concentration-dependent manner (from 1 M to 0.01 M KCl) (Figure 4B). It was not possible to retain KCl-charged MjK2 liposomes with the same high signal level prepared without BaCl₂. Interestingly, the potassium release measurements from preparations with internal cAMP had two effects. At pH 8 potassium release was less than observed with at pH 4, but at internal pH 4, cAMP caused a drastic decrease of the signal as observed only from preparations which obtained when liposomes prepared without external BaCl₂, therefore it could be that cAMP may arrest MjK2 in an open configuration (Figure 4C). The results of Figure 4 as differences from baseline and plateau are summarized in Table 1.

The structure of MjK2 aligns with the structures of members of the RCK-channel family, but the signature for divalent cation binding site is different

The structure of MjK2 is not available, which may give an explanation on the structural level for the observed different effects on the channel activity. Amino acid alignment revealed to many differences, therefore the MjK2 amino acid sequence was tested by Swiss Model software. Sufficient structural homology was found with the transmembrane domains of MjK2 which superimposed on the MthK channel structure (Figure 5A). However, TM2 of MjK2 is found on the cytoplasmic side in a slightly different position than in the MthK structure. Position W52 in MthK is replaced with F38 in MjK2 (Figure 5A). The effect of aromatic substitutions at related positions (Trp-67 to Phe-67 and Tyr67) in KcsA has been shown previously [24]. In

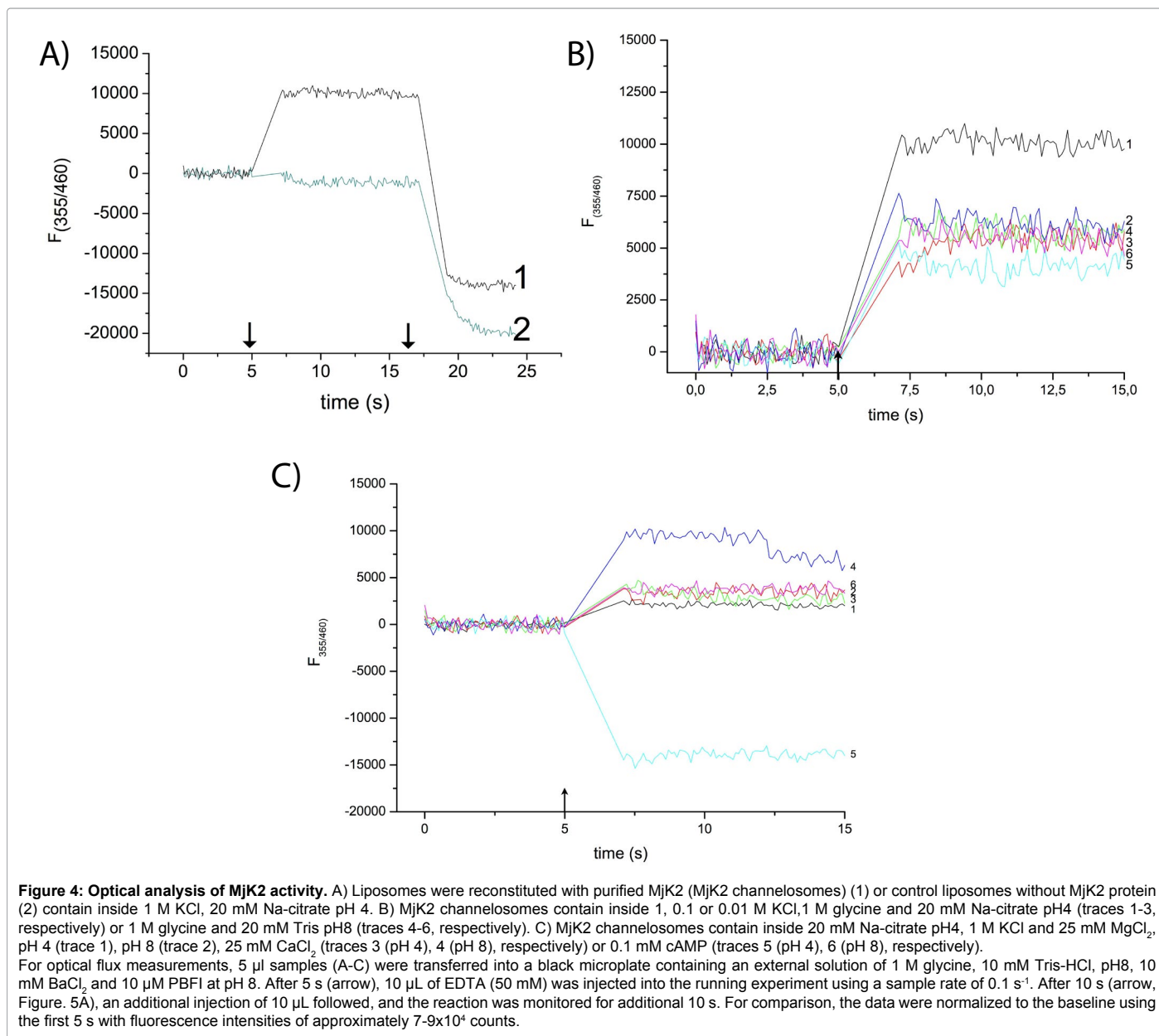
this case, W67Y leads to C-type inactivation, perhaps because of the collapsed filter structure.

Likewise, the MjK2-RCK domain superimposed on three other RCK gating rings of MthK (Figure 5B; magenta) and on the human K⁺ channels *hslol* (Figure 5B; light blue) and *hslol3* (Figure 5B; yellow). Partial segments of the model of MjK2 (Figure 5B, orange) superimpose with all α -helices and β -sheets on helix α F of MthK, but data for α G and Ca²⁺ sites 1-3 (Ca²⁺ bowl) that occur in MthK were not found in MjK2. This is also in agreement to other structural data, since D184 is in MthK a position important for divalent cations or Ba²⁺-binding, which does not exist in MjK2 [1,24]. He observed inhibitory effect is probably due to the binding to the pore as observed for KcsA. The next partial alignment begins with β G and can be superimposed with the remaining portions of the protein onto helix α J. The RCK structures of the closed *hslol* and open *hslol3* (partial segments of *hslol* that did not contribute to alignments were removed for visualization) agree with MthK for the first 250 amino acids. However, over the full length and after α G, there was an extra helix α G', which did not occur in MthK. It was argued that RCK-gated human *hslol* and *hslol3*, sperm cell K⁺ channels, are different in their pH and Ca²⁺ activation. Additionally, *hslol3* is pH sensitive and may contain a pH sensor [26]. The RCK's of both *hslol* channels have two lysines, 332 and 331, at the entry (Figure 5B; K331_{slo3} and K343_{slo1}). Interestingly, at a similar position before the N-lobe of the RCK domain with β A begins, MjK2 also has a lysine (K107) in the linker region at the end of a lysine and arginine sequence with seven lysines (K89, K97, K98, K100, K102, K104 and K107) and one arginine (Figure 5A).

Discussion

MjK2 is a RCK channel with atypical properties as suggested previously [15] and shown in this study using structural alignment with the model of MjK2. Ion channels can have two states, closed or open, but several intermediate steps can exist between these states [6]. The data shown here reveal that MjK2 is activated by pH, but additional various activating/inactivating regulatory steps occur.

MjK2 was activated in electrophysiological experiments only by low pH, but in the optical assay, activation occurred also with the addition of Ca²⁺ at pH 8 while MgCl₂, pH 8 alone or cAMP did not elicit significant channel activity. However, cAMP at low pH reduced background release level to that of liposomes. This finding also indicates that MjK2 does not behave similarly to MthK or Tvok channel [4-7,10]. MjK2 did not show enhanced stability or single channel activity when divalent cations present. In the presence of Ca²⁺ or low pH, dimers of the MjK2 protein were formed, but those conditions did not elicit the higher oligomeric forms that were observed for MthK [4,6]. In addition, a stabilization effect on the isolated RCK (start codon MKNK) domain with Ca²⁺ was also not observed (data not shown). A structural comparison, to other K⁺ channels similar to both, MthK and *hslol3*, showed that MjK2 has a lysine at a similar position (K107) [25]. In *hslol3*, K332 is a putative pH sensor, through which lower proton concentrations increase the relative current [25]. In contrast, the linker of MjK2 could have the opposite effect on gating. The linker between TM2 and RCK of MjK2 has seven lysines, and MthK has two. It is unknown whether this difference is responsible for variations in the activation behavior or whether the activation differs because of the postulated charge destabilization of RCK with the lipid phase that occurs at high KCl concentrations [15]. In contrast, the presence of high internal Ca²⁺ levels at pH 8 had the same effect as pH 4 and high KCl in optical measurements; although no stabilization favoring the



formation of a tetrameric channel was observed with Ca²⁺, which does occur for MthK [4-8]. In addition, the stability experiments revealed that in the presence of low pH or Ca²⁺, RCK dimers were observed, which form the locked state in MthK [4,5]. However, this result indicates that because of the stabilization of the membrane portion, channel tetramers enabled the surface charges as postulated by [15] that contribute to the channel activity in the presence of pH 8 and Ca²⁺. Taken together, the channel function is restored without the extra soluble RCK domain and it could be that the additional RCK can modulate the channel function by a weak interaction.

Moreover, the selectivity filter responsible for K⁺conductance corresponds to a collapsed structure in mutated KcsA [22], which may not be able to conduct potassium. This hypothesis was not in agreement with the experimental findings, although single channels were only recorded at low pH and high potassium chloride. *M. jannaschii* is a marine organism that survives in high external sodium

chloride concentrations (~660 mM). Under these conditions, the collapsed orientation of the filter may hinder the entrance of sodium. Consequently, the MjK2 channel is mostly inactivated, and activation may occur when the internal potassium concentration is drastically increased.

Conclusion

MjK2 is a RCK channel, but in contrast to other MthK Ca²⁺-activated K⁺ channels becomes activated by low pH, but may have an unclear effect with nucleotides on transport studies. An unknown modulatory Ca²⁺ activation at pH 8 was observed, although a typical binding site could not be postulated. It is possible that nucleotides are modulators of the channel function. Additionally was shown that PBFI, a potassium sensing dye, is a useful tool for throughput applications in screening of K⁺ channel activities as tested by several conditions to influence activity of MjK2.

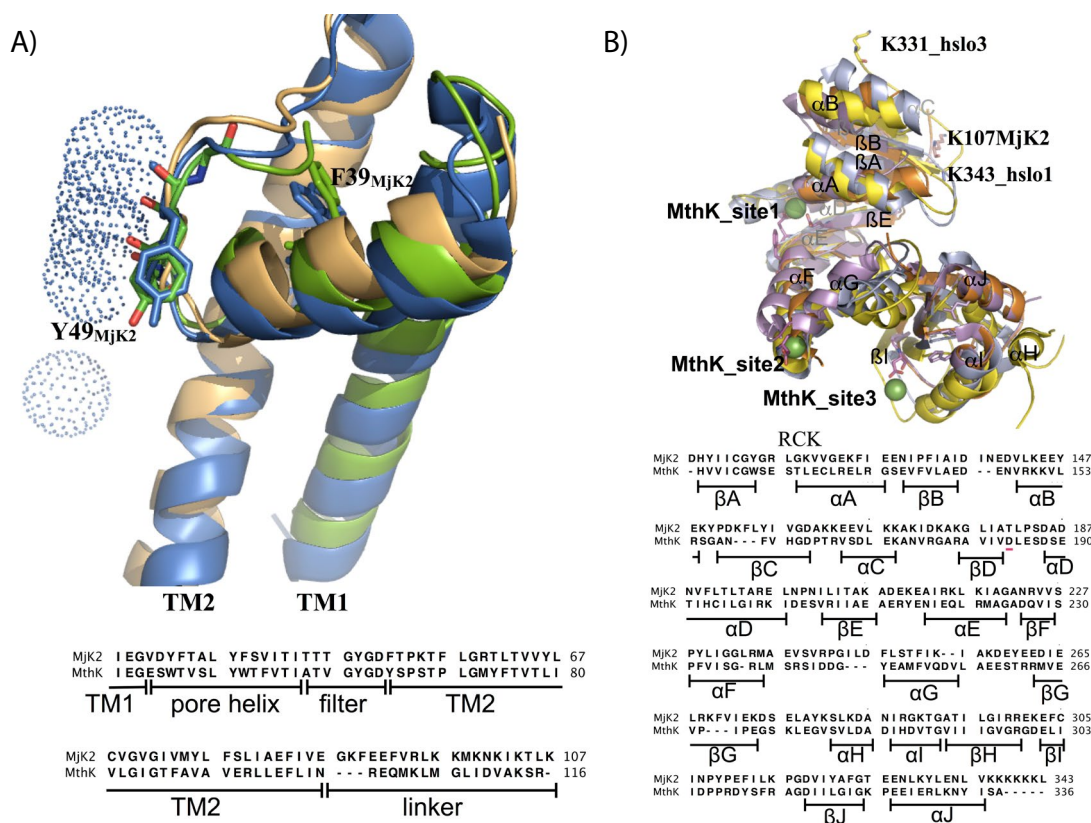


Figure 5: a) Structural alignment of MthK and the modeled MjK2. The transmembrane domain of MjK2 (green) modeled based on template 3LDC (1.45 Å) using Swiss Model software [29-31] and aligned with the MthK structure (blue; 3LDC) obtained from www.pdb.org. The potassium-selectivity filter, F39 (MjK2) and W52 (MthK) are illustrated using sticks, whereas potassium is given as spherical marbles. The amino acid sequence alignment is shown below.

b) Structural alignment of the RCK domains of MthK, *hsl01*, *hsl03* and modeled MjK2. The RCK domain of MjK2 (orange) modeled based on the template InqE (3.30 Å) using Swiss Model software [29-31] aligned with the gating rings of MthK (magenta; 3RBZ (8)), *hsl01* (yellow; 3NAF (25)) and *hsl03* (pale blue; 3U6N (26)) obtained from www.pdb.org. The structures were edited and visualized using Pymol software. The position D184 in MthK is indicated by a red line.

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