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Negative and positive temperature dependence of potassium leak in MscS mutants: Implications for understanding thermosensitive channels

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ABSTRACT

Bacterial mechanosensitive channel of small conductance (MscS) is a protein, whose activity is modulated by membrane tension, voltage and cytoplasmic crowding. MscS is a homoheptamer and each monomer consists of three transmembrane helices (TM1-3). Hydrophobic pore of the channel is made of TM3s surrounded by peripheral TM1/2s. MscS gating is a complex process, which involves opening and inactivation in response to the increase of membrane tension. A number of MscS mutants were isolated. Among them mutants affecting gating have been found including gain-of-function (GOF) and loss-of-function (LOF) that open at lower or at higher thresholds, respectively. Previously, using an *in vivo* screen we isolated multiple MscS mutants that leak potassium and some of them were GOF or LOF. Here we show that for a subset of these mutants K^+ leak is negatively (NTD) or positively (PTD) temperature dependent. We show that temperature reliance of these mutants does not depend on how MS gating is affected by a particular mutation. Instead, we argue that NTD or PTD leak is due to the opposite allosteric coupling of the structures that determine the temperature dependence to the channel gate. In PTD mutants an increased hydration of the pore vestibule is directly coupled to the increase in the channel conductance. In NTD mutants, at higher temperatures an increased hydration of peripheral structures leads to complete separation of TM3 and a pore collapse.

1. Introduction

Ion channels are allosteric proteins that are able to integrate various stimuli, *e.g.*: the opening of BK K^+ channels is modulated by voltage and Ca^{2+} [1]; HCN channels are modulated by voltage and cyclic nucleotides [2] and TRP channels are modulated by voltage and temperature [3,4]. The gating of bacterial mechanosensitive (MS) channel MscS from *Escherichia coli* is modulated by membrane tension [5], voltage [6] and osmotic stress [7,8]. It is, therefore, crucial to know how various stimuli affect processes that lead to channel activation. MscS is a homoheptamer (Fig. 1A, B) of monomers whose transmembrane part is built of three helices: peripheral TM1 and TM2 that surround pore forming TM3 (Fig. 1C) [9]. The channel opens upon membrane stretch as a result of straightening of TM3a and TM3b helices (Fig. 1C) and dissociation of TM3b from ß-domain, which forms cytoplasmic part of the channel [10,11]. It was proposed that the decoupling of TM1/2 from TM3 results in the expansion of crevices between these structures and channel inactivation [6,12]. Both channel pore and the peripheral crevices are highly hydrophobic [12,13].

Ion channel gating is in many instances based on hydrophobic water repulsion. This mechanism was first recognized in simple carbon nanopores [14,15] and was postulated for several channels including MscS [16], nAchR [17], GliC [18] and K^+ channels [19]. It is supported by the existence of conserved cavities or rings of hydrophobic amino acids inside channel pores [16], which hydrophilic substitutions lead to mutants with increased open probability [20]. Such MscS mutants, called gain-of-function (GOF), exhibit low threshold pressures or spontaneous openings leading to solute leak and cell death [11,20]. This is in contrast to loss-of-function (LOF) mutants exhibiting increased threshold pressures [21,22]. It has been shown that the GOF phenotype of MscS-G98S (Fig. 1D) is a result of increased pore vestibule hydration [13]. On the other hand the LOF phenotype of MscS-V65S (Fig. 1D) is due to the separation of TM1/2 from TM3 pore helices (Fig. 1C), however, no hydration of hydrophobic peripheral crevices was observed during MD simulations [12].

In our previous work we used a K^+ uptake-deficient strain and observed its growth in various K^+ concentrations [11]. This method allowed us to isolate a number of K^+-leaky MscS mutants and determine the threshold of K^+ concentration required for the growth of a particular MscS mutant [11]. We were able to ascribe clear GOF or LOF phenotypes to a number of K^+-leaky MscS mutants (Fig. 1D), *e.g.* GOF phenotypes were exhibited by I97N and A98S pore mutants. However, general location does not seem to be a prerequisite for GOF phenotype *e.g.* A94D, one of the mutants from the pore region did not exhibit

Abbreviations: CP, specific heat capacity; GOF, gain-of-function; LOF, loss-of-function; MS, mechanosensitive; NTD, negative temperature dependent; PTD, positive temperature dependent; TM, transmembrane helix

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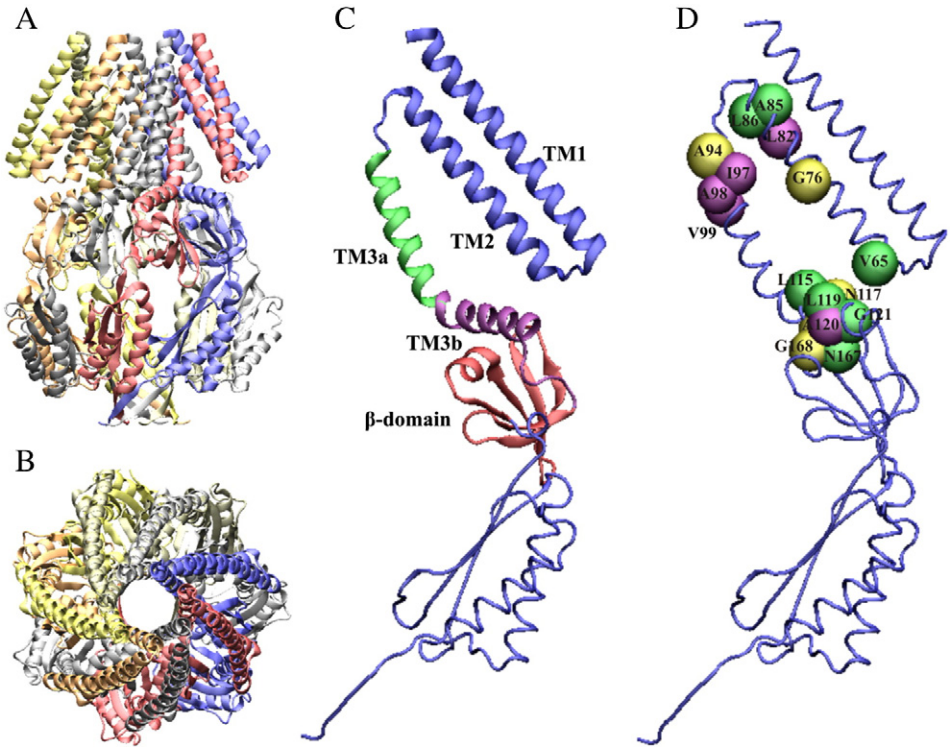

Fig. 1. The crystal structure of MscS heptamer (A., B.) and its monomer (C., D.) (PDB ID: 2OAU). The side (A.) and the top (B.) views of MscS with each monomer shown with a different color. C. MscS monomer with its domains marked: TM1/2 (blue), TM3a\b (green\purple), ß-domain (red). D. MscS monomer, the positions of the amino acids altered in K^+-leaky mutants isolated in (11) are shown as balls. The substitutions that result in the GOF or LOF phenotypes are marked by purple and green, respectively. The amino acids that cause K^+ leak without any additional phenotype are shown in yellow.

GOF. Interestingly GOF phenotype was exhibited by the mutants: V99E on TM3 located slightly outside of the pore, and L82E on the peripheral TM2, indicating rotation of the TM3 helix during the opening of the channel [23]. On the other hand, LOF phenotype, was a result of changes of the following amino acids: V65D, A85T, L86N, V96D, L115Q and L119D located in the peripheral crevices. However, no LOF phenotype was observed in the G76D mutant even though G76 is located in these crevices.

In general, the transition from the closed to the open channel might be the result of minute conformation changes involving movement of amino-acid side chains [24]. Consequently, channel gating is influenced by protein thermal motions and an increase in temperature can shift the equilibrium towards the open channel state [25]. For most channels changes in the gating evoked by heat within the physiological range are insufficient to open them. Transient receptor potential (TRP) channels are those of exception. The temperature coefficient Q_{10} of TRP channels exceeds 100, which translates to the enthalpy in the order of 100 kJ/mol [26]. Enthalpy of this magnitude is comparable to the total heat of unfolding of small proteins like lysozyme [27], but not consistent with the relatively small conformational changes typical for the ion channel gating. It was speculated therefore, that the thermal sensitivity of TRP channels can arise from changes in the temperature-induced membrane fluidity [28]. However, other numerous facts support the hypothesis that thermosensitivity is an intrinsic property of the channel proteins. It has been shown that certain extramembranous domains within TRPV and TRPA channels are responsible for the thermal sensitivity, and the transfer of these domains into non-thermosensitive homologs is sufficient for the recovery of heat sensitivity [29,30]. Moreover, thermosensitivity has been detected in structurally related but functionally distinct voltage-activated Kv channels and certain mutations in voltage sensing domains increase dramatically the temperature dependence of these channels [31,32].

We wondered whether the MscS channel activity is modulated by temperature and if so, whether mutations that change properties of the channel pore can influence the temperature sensing of MscS. Here we report that some MscS mutants that change mechanosensitivity of the channel modulate also its thermosensitivity.

2. Materials and methods

2.1. Media

Growth assays were performed in the defined media. The K115 media consisted of the following: K_2HPO_4, 46 mM; KH_2PO_4, 23 mM; $(NH_4)_2SO_4$, 8 mM; $MgSO_4$, 0.4 mM; $FeSO_4$, 6 μM; sodium citrate, 1 mM; thiamine hydrochloride, 1 mg/l; and glucose, 0.2% (w/v) [33]. For K0 medium, equimolar sodium phosphate was used to replace potassium phosphate. Suitable mixed proportions of K0 and K115 were used to create a medium with intermediate K^+ concentrations. In all media, ampicillin was used at 100 μg/ml. Solid media contained 1% [w/v] agar.

2.2. Strains

E. coli strain deficient in potassium transport systems TK2446 (*F − thi rha lacZ nagA Δ(kdp FAB)5 trkD1 trkG(kan) trkH(cam)Δ(trkA-mscL)*) and LB2003 (*trkAkup1 (trkD1) kdpABC5 rpsL metE thi rha gal*) were kindly provided by Professor Brad S. Rothberg (Temple University School of Medicine, Philadelphia) and Professor Evert P. Bakker (University of Osnabruck, Germany). *E. coli* strains MJF429 (*ΔmscK::kan ΔmscS*) and MJF465 (*ΔmscK::kan ΔmscS ΔmscL::Cm*) were kindly provided by Professor Ian R. Booth (University of Aberdeen, Aberdeen, UK).

2.3. Phenotypic analysis

The construction of plasmids carrying K^+ leaky mutants of MscS was described earlier [11]. For phenotypic analysis the plasmids expressing mutated MscS were transformed into TK2446 or LB2003 strain and grown overnight in K115 media at 37 °C in 96-well plates. The individual

cultures were replica plated with 96-pin lids (Nunc™ TSP Screening) into square dishes (Nunc™ OmniTray™) containing solid media with various K^+ concentrations. These dishes were incubated overnight at various temperatures.

2.4. Electrophysiological recordings

Single channel recordings were obtained from inside-out excised membrane patches derived from MJF465 (MscS-V65D) or MJF429 (remaining MscS mutants) strains. The experimental procedure and equipment used were the same as described earlier [11]. Pressure pulses, at constant pipette voltage +15 mV, were applied with a high speed pressure clamp (HSPC-1). The temperature was controlled by a custom made system based on Peltier elements. Bath solution contained 400 mM sucrose, 200 mM KCl, 90 mM $MgCl_2$, 4 mM $CaCl_2$, and 5 mM HEPES, pH 7.2. The pipette solution was the same as the bath solution except it lacked sucrose.

Intervals of 3 min or longer were maintained between consecutive applications of pressure pulses. Data were acquired (with a sampling rate of 5 kHz), filtered at 2 kHz, and analyzed using pCLAMP10 software (Molecular Devices).

2.5. Immunoblotting

MscS mutants bearing a C-terminal 6His-tag were expressed in MJF429 strain of *E. coli*. The cells were lysed in PBS supplemented with 1 mM PMSF by sonification for 3 min. Protein concentration was determined in the presence of 1% SDS with BCA protein assay kit (Pierce). Protein concentration was adjusted and samples mixed with LDS gel loading buffer on ice [34]. Equal amounts of proteins were separated by LDS-PAGE at 4 °C [34], transferred onto PVDF membranes, and then immunoblotted with antibody against 6His-tag (GenScript Co.). The blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Pierce) for 1 h, and chemiluminescence was detected with an ECL system (GE Healthcare).

2.6. Data analysis

The relative growth of K^+-leaky mutants at each temperature was calculated as a ratio of OD_{600} of cultures grown for 24 h in the media with a limited K^+ concentration and 50 μM IPTG, and OD_{600} of cultures grown for 24 h in 115 K media with 1 mM IPTG.

In order to quantify channel thermal response, the temperature coefficient of the maximal current (Q_{10I}) [31] was calculated as:

$$Q_{10I} = \left(\frac{I2}{I1}\right)^{10/[T2-T1]},$$

where $I1$ is a maximal current amplitude at 18 °C ($T1$), and $I2$ is a maximal current amplitude at 33 °C ($T2$).

Similarly, the temperature coefficient of the inactivation $Q_{10\tau}$ was calculated according to the formula:

$$Q_{10\tau} = \left(\frac{\tau 2}{\tau 1}\right)^{10/[T2-T1]},$$

in which $\tau 1$ and $\tau 2$ denote time constants of inactivation measured for channels recorded at 18 °C and 33 °C, respectively.

Time constants $\tau 1$ and $\tau 2$ were derived by fitting single exponential decay function to individual current (I) traces using the function:

$$I = \sum_{i=1}^{n} e^{-t/\tau i}.$$

All statistical data are given as mean ± SEM. For statistical analysis Student's 2-tailed t-test with groups of unequal variances was used. Protein structures were visualized using VMD [35].

3. Results

The growth of the K^+ uptake-deficient strain is a very sensitive bioassay of channel permeation [36,37]. Previously, we used this assay to isolate MscS K^+-leaky mutants [11]. The threshold of K^+ concentration, required for the growth of particular MscS mutant, was determined within 1–2 mM resolution. For this study, we screened the growth of previously isolated single MscS mutants and the experiments were carried out in four K^+ concentrations (2, 4, 6, 115 mM) and in temperatures varying from 18 °C to 42 °C. We found that for a subset of mutants the growth on plates with media containing a given concentration of K^+ was dependent on temperature (Fig. 2). Some mutants tended to grow at lower temperatures and did not grow at higher temperatures, thus they exhibited a negative temperature dependent growth (NTD), whereas other mutants grew only at higher temperatures exhibiting a positive temperature dependent growth (PTD). Among 35 tested mutants we found nine of the NTD phenotype and four of the PTD phenotype. Mutants of NTD phenotype were V65D, G76D, A94D, I97N, N117K, L118P/Q, A120D and G168D, while those of PTD phenotype were L82E, A98S, V99E and G121D (the positions of mutations are shown in Fig. 1D).

To confirm the validity of the data from the screen on the solid media, we chose four mutants V65D, G76D, L82E, A98S exhibiting pronounced growth temperature dependence to study their growth in liquid media. Growth curves for four concentrations of K^+ (4, 8, 12, 115 mM) for each strain were obtained in three temperatures (Fig. 3). V65D and G76D mutants, both on solid media and in liquid cultures exhibited NTD phenotypes whereas the two other mutants L82E and A98S exhibited in both growth tests PTD phenotype. It is worth noting that both NTP strains grew well in 24 °C while in 37 °C no growth was observed. Analogous (reversed) temperature dependence (13 °C difference between the optimal temperature of growth and the temperature that caused its cease) was observed for PTD strains. Calculation of the growth in potassium limiting media in the relation to the growth in 115 K media after 24 h of incubation at 24 °C, 30 °C and 37 °C allowed us to compare more quantitatively the temperature dependence of the PTD and NTD mutants (Fig. 3C).

To exclude that NTD or PTD phenotypes are due to the protein instability at extreme temperatures we analyzed the amount of MscS heptamers of chosen MscS mutants grown at 24 °C or 37 °C by LDS-PAGE (Fig. 4) [34]. We did not observe significant differences between the wild type and the MscS mutants therefore differences in the protein stability or oligomerization are unlikely to be responsible for NTD or PTD phenotypes.

Since in MscS-L82E mutant the GOF phenotype is apparently associated with the positive temperature dependence and in MscS-V65D mutant the LOF phenotype is associated with the negative temperature dependence, we wondered whether the MscS mechanosensitive activity of these mutants is affected by temperature in the same way as the K^+ leak detected *in vivo*. Therefore, we used a patch-clamp technique to record activities of the wild-type and of the mutants of MscS at two different temperatures (18 °C and 33 °C) (Fig. 5). Due to residing in a permanently inactivated state, V65D mutant exhibits the LOF phenotype [11], and only a residual MS activity at extremely high pressures can be observed (Fig. 5A, upper row). For both V65D and L82E mutants the higher temperature resulted in a faster rate of adaptation (Fig. 5A, two upper rows). This could indicate that inactivating component of the MS current depends on temperature but does not determine the opposite temperature dependence of K^+ leak of these mutants. Inactivation is a part of a complex process called adaptation, which involves conformational changes of the channel protein dwelling into an inactivated state, and a relaxation of membrane tension by lipid flow [38]. The relation between these two processes is complex since some of the MscS mutants do not adapt at all [10,11,39]. To determine whether increased fluidity of the membrane is coupled to the channel conformational changes we recorded the MscS wild-type activity using two-

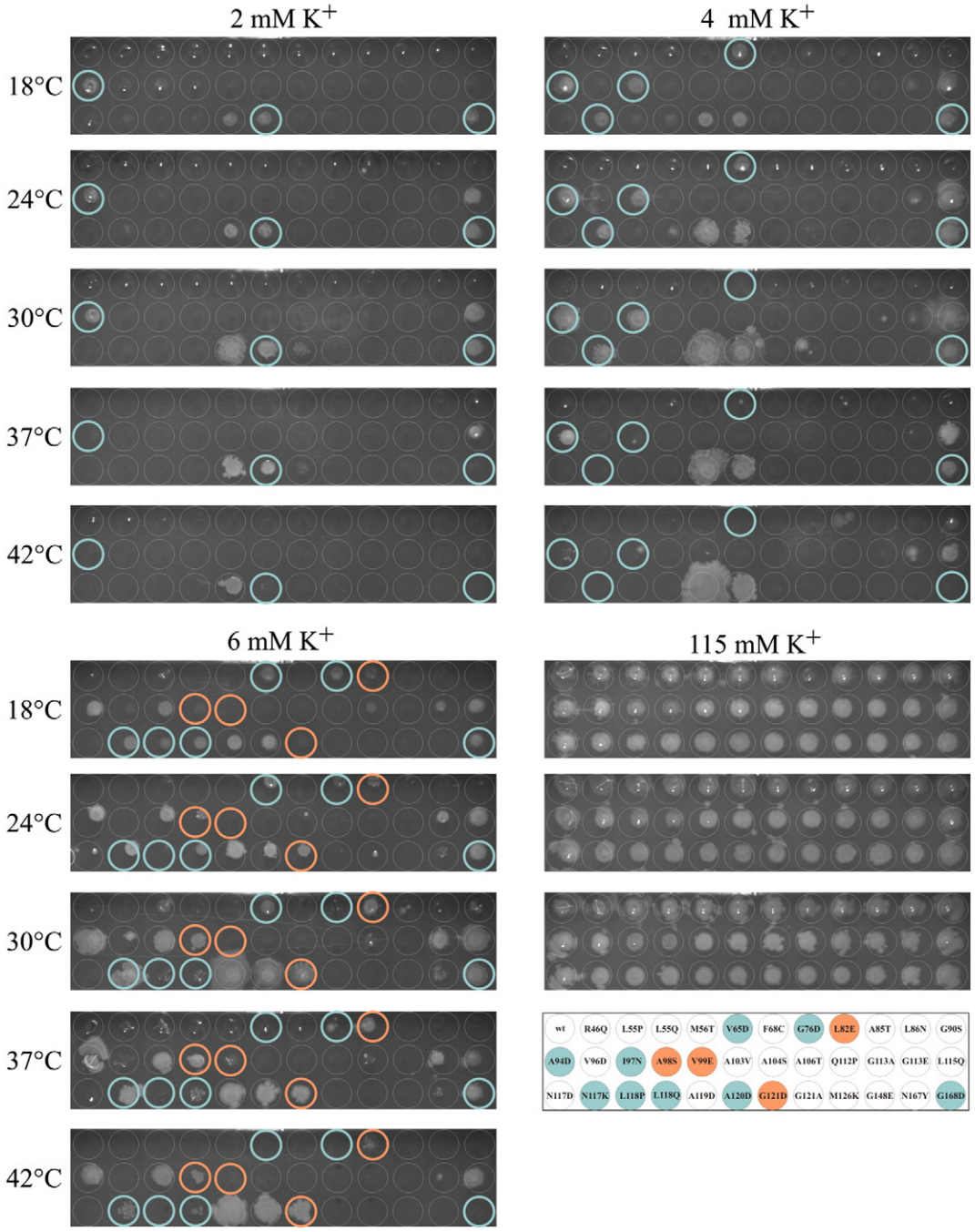

Fig. 2. The temperature and K^+-concentration dependence of growth on solid media for a set of K^+-leaky MscS mutants. For a subset of mutants the negative (NTD, cyan) and the positive (PTD, orange) temperature dependent growth is observed. The NTD mutants exhibit loss of growth at higher temperatures for a given K^+ concentration, whereas the PTD mutants exhibit gain of growth at higher temperatures for a given K^+ concentration. Bacteria were grown on minimal solid media containing growth-limiting K^+ concentrations with 50 μM IPTG. Under these conditions the expression of the mutants was not toxic as proven on the media with non-limiting K^+ (115 mM) with 50 μM IPTG.

pressure-step protocol, in which the number of non-inactivated channels could be determined by a second saturating pressure step (marked by asterisks in Fig. 5A, third row) [6]. We observed that similarly to V65D and L82E mutants the MscS-wt channels adapted faster at the higher temperature. The application of the second pressure pulse demonstrated that the number of inactivated channels was increased (Fig. 5A, third row). We asked whether inactivation is the only channel state affected by temperature. To answer this we used G113E mutant, which lacks inactivation (Fig. 5A, bottom row). The number of G113E channels was substantially lower at 33 °C showing that mechanosensitive opening is affected by temperature. In order to look at the effect of temperature more quantitatively we calculated the temperature coefficients: Q_{10I} and $Q_{10\tau}$. Q_{10I} was calculated for the maximal current (I_{max}) for the wild type and for the G113E mutant, and $Q_{10\tau}$ for the wild-type time constant of inactivation (τ) in different pressures. We noticed a high variability of both coefficients between patches (Fig. 5B). This variability could be explained partially by different patch geometries resulting in shifts of threshold pressures in the patches [40]. In spite of that Q_{10I} and $Q_{10\tau}$ values were consistently <1 in all patches. This indicates for negative temperature dependence of both the channel opening and the inactivation. The temperature coefficient Q_{10I} is pressure dependent and we noticed higher Q_{10I} values for higher pressures (Fig. 5B). On the other hand $Q_{10\tau}$ did not exhibit clear pressure dependence (Fig. 5B). Therefore, we calculated mean $Q10_\tau$ of all the values for the wild-type MscS (0.37 ± 0.13, $n = 9$),

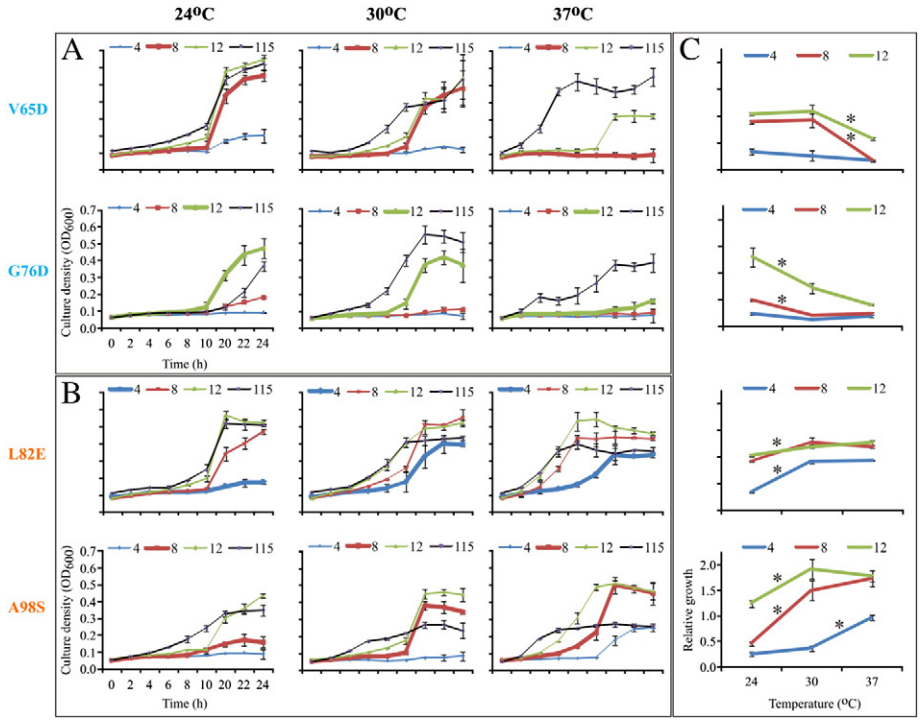

Fig. 3. The temperature and the K^+-concentration dependence of bacterial growth in the liquid media. A. B. Growth curves for two NTD (A.) and two PTD (B.) mutants in four K^+ concentrations (4, 8, 12, 115 mM) are presented. The signature growth curves at a given K^+ concentration in which a clear temperature dependence is visible are shown with thicker lines. Data are shown as means ± SEM. from three independent cultures for each growth condition. C. The relation between temperature and the relative growth of the NTD and PTD mutants from (A.) and (B.). The calculation of the relative growth is described in Section 2.6. Data obtained in three independent experiments are shown as means ± SEM. K^+ concentrations (in mM) are indicated with the legends above each graph. Asterisks (*) mark these parts of the graphs for which statistical significant differences ($p < 0.05$) were found. For each of the A., B. and C. panels scales are shown for one graph only.

for the L82E mutant (0.54 ± 0.05, n = 4), and for the V65D mutant (0.57 ± 0.06, n = 4). The rate of inactivation of the G113E mutant was to low to reliably calculate time constants. All the $Q_{10\tau}$ values are similar, therefore we favor the hypothesis (see Section 4) that in all MscS variants a higher membrane fluidity, hence tension relaxation by lipid flow in the pipette, dominates the temperature-dependent modulation of mechanosensitive activity. At elevated temperatures MS activities of the channels are less coupled to the membrane mechanics. Mechanosensitivity of MscS is therefore affected by temperature in a nonspecific way (*via* lipids) and does not determine the NTD and PTD phenotypes.

4. Discussion

We showed that the influx of K^+ in MscS potassium leaking mutants exhibits a temperature dependence. We showed that the mutants V65D and G76D with altered amino acids located within the crevices between TM1/2 and TM3 exhibited the NTD phenotype. Alternatively, the mutants L82E and A98S with altered amino acids located around the outer pore vestibule exhibited the PTD phenotype. We also showed that GOF and LOF phenotypes of other mutants do not correlate with NTD and PTD phenotypes. Our results also indicate that MscS channel and MscS K^+ leaky mutants are modulated by temperature in a distinctive way.

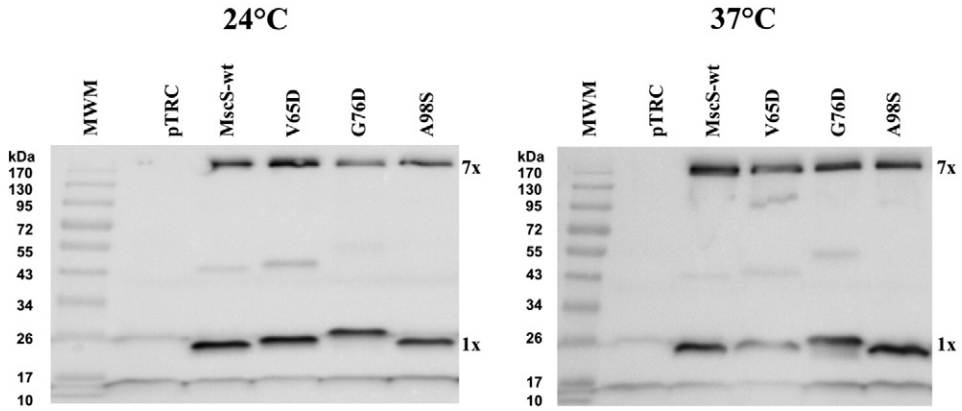

Fig. 4. The growth temperature does not influence the stability and the oligomeric state of the MscS mutant proteins. Cell lysates derived from bacteria expressing the wild type or the mutants of MscS tagged with C-terminal 6xHis epitope were separated by LDS-PAGE (34) and probed with anti-6His antibody. The positions of monomers and heptamers of MscS are marked by "1x" and "7x" respectively.

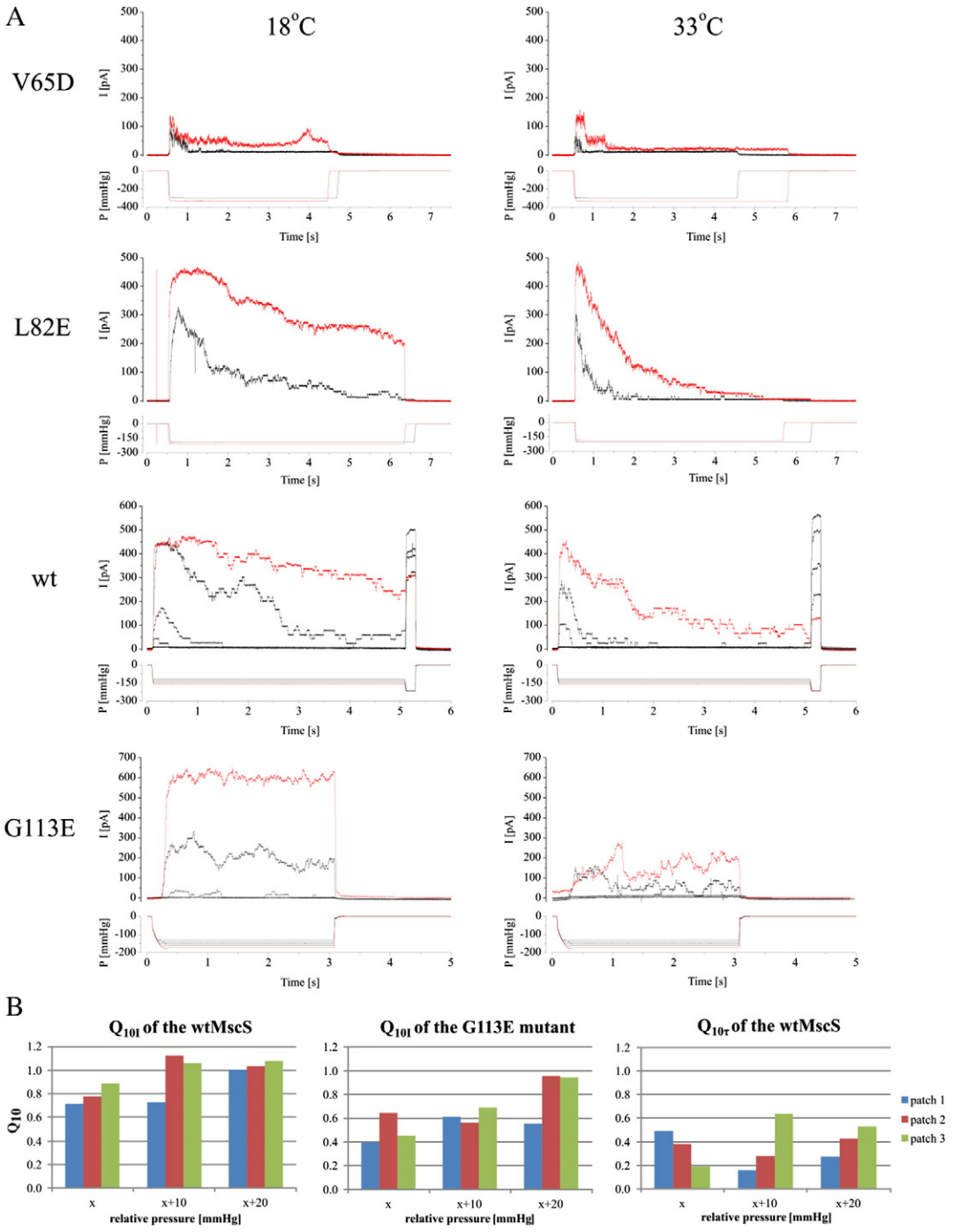

Fig. 5. Patch-clamp recordings of the NTD (V65D), the PTD (L82E), the non-inactivating mutant (G113E) and the wild type MscS. The temperature sensitivity of the K^+ leak in the NTD and the PTD mutants does not depend on the mechanosensitive component of the channel gating. A. Both the V65D and the L82E mutants similarly to the wild-type MscS exhibit faster adaptation at 33 °C than at 18 °C. Fewer channels of the non-inactivating G113E mutant open at 33 °C as compared to 18 °C (bottom row). In each row recordings marked in red show activity of channels at the highest pressure applied to the same membrane patch at 18 °C and 33 °C. Each experiment was repeated at least three times. B. The temperature coefficients Q_{10I} and Q_{10T} of the wild-type MscS and Q_{10I} of the G113E mutant for three consecutive pressure applications in three different patches.

Membrane proteins are tightly coupled to lipid dynamics [41]. This coupling is especially pronounced in the case of bacterial MS channels that directly sense membrane physical state [42–45]. Indeed, all known modulators of these channels affect properties of lipid membranes [44–46]. Lipid membranes change their properties dramatically in response to changes in temperature shifting from gel to liquid-crystalline phase at a characteristic transition temperature Tm. Tm of biological membranes, including that one of *E. coli* lies always 10 °C–15 °C below the growth temperature [47]. It was modeled that in liposomes an increase in temperature results in an increase in the membrane tension up to Tm and further temperature increase results in a decrease in tension [48]. Increased tension is a trigger for MscS opening. Our recordings showed that the MscS mechanosensitive activity at 18 °C and 33 °C were below and above the expected transition temperature (23 °C–28 °C) [49], respectively. Therefore, it was difficult to predict whether tension was different in both temperatures. For *E. coli* grown at 37 °C less than 40% of the membrane is fluid in temperatures below 20 °C, and more than 90% of the membrane is fluid at temperatures higher than 30 °C [50]. It indicates that at higher temperatures an increased lipid flow and a tension relaxation within a membrane patch are responsible for the

temperature dependence of the MS current of MscS [51]. However, we cannot exclude that the temperature dependence is due to changes of specific heat capacity of the MscS channel protein itself (see below).

Our experiments indicate that the temperature dependence of the mechanosensitivity of MscS is different from the temperature dependence of *in vivo* K^+ leak *via* MscS mutants. This conclusion comes from a number of observations. First, K^+ leak *via* MscS mutants is independent on the mechanosensitivity of MscS, since low- and high-threshold mutants leak K^+. In an extreme case only the residual MS activity of MscS-V65D is observed at nearly lytic membrane tensions and still this mutant can support growth on low K^+. Consequently, PTD and NTD phenotypes do not correlate with GOF and LOF phenotypes, which can be supported by the following observations: MscS-I97N exhibits both GOF and NTD phenotypes, while MscS-A98S is GOF and PTD; V65D exhibits both LOF and NTD phenotypes, while G121D is LOF and PTD (Table 1). Further, the electrophysiological characteristics of V65D and G168D mutants are entirely different, even though both mutants exhibit NTD phenotype. V65D inactivates extremely quickly, whereas G168D does not inactivate at all [11]. As it has been shown bacteria adjust membrane lipid composition to the growth temperature in such a way that membrane remains in a fluid state during cell growth [52]. In *E. coli* grown at 37 °C the lipid transition temperature Tm is around 28.5 °C and falls to 10 °C for cells grown at 17 °C [53]. It seems, therefore, unlikely that NTD and PTD phenotypes arise from different membrane fluidity at low and high temperatures of growth. It has been shown that MscS co-localizes with cardiolipin at the cell poles of *E. coli* and that cardiolipin content increases at higher temperatures [54]. Given that it has been shown that cardiolipin directly affects gating of MscS by increasing the closing rate of the channel [55] it is possible that in the case of MscS and its specific interaction with cardiolipin the lipids could also contribute to the NTD phenotype at higher temperatures. On the other hand, apparently none of the substituted amino acids face lipids, *i.e.* protein–lipid interface is the same or similar in both PTD and NTD mutants. Therefore, we believe that elevated binding of cardiolipin to MscS at higher temperatures should affect both types of mutants to the same extent. Putting aside all abovementioned ambiguities our data indicate that K^+ leak is temperature- but not mechano-sensitive.

What is then the mechanism responsible for K^+ leak? In MscS, all the mutations that render K^+ leak are focused around TM3 helices. TM3 movement is restricted by two structures: TM1/2 that binds TM3 in the closed and the open state [56] and the ß-domain that binds TM3 in the closed and in the inactivated state [7,11]. The movement of TM3 helices is probably less restricted in K^+-leaky mutants allowing for minute K^+ inward currents through the channel pore which we were able to detect by the growth assays (Fig. 6).

Bearing in mind that the decoupling of TM3 pore helices from surrounding structures of the TM1/2 "mechanosensor" and/or the ß-domain is responsible for the K^+ leak we also think that it could also be the key to the temperature sensitivity of MscS mutants. This mechanism would be strikingly similar to the mechanism of thermal sensitivity of K^+ channels. It was recently revealed that Shaker Kv channels exhibit thermal sensitivity in a narrow voltage range, at which the channel voltage dependence is weak due to separation of the voltage sensor from the pore domain [31]. The decoupling of the voltage sensor is also a result of mutations in ILT and V2 Shaker mutants and both of them exhibit increased thermal sensitivity. Strikingly, thermal sensitivity of ILT and V2 mutants is opposite, being positive and negative, respectively. The opposite temperature dependence was observed in closely related TRP channels [57] and recently it was proposed that the opposite allosteric coupling is responsible for this phenomenon [4]. Alternatively, negative or positive changes in heat capacity could explain variety of temperature-sensitive phenotypes between closely related channel proteins [26,32]. Calorimetric measurements show that hydration of hydrophobic residues is associated with a positive change in the specific heat capacity (ΔC_P), while that of polar/charged residues is associated with the negative ΔC_P [58,59]. Protein unfolding accompanied by water exposure of hydrophobic amino acids is therefore ultimately associated with changes in the specific heat capacity, which in turn determines the free energy dependence on temperature [60,61]. Based on this principle the mutant Shaker Kv channels were constructed with single amino acid substitution and these mutants exhibited either cold (exposure of hydrophobic residue) or heat (exposure of polar residue) sensitivity [32]. Opening of the MscS channel is associated with water exposure of the hydrophobic pore [13]. It is therefore plausible that the apparent cold sensitivity of the MscS mechanosensitive activity observed in the patch-clamp experiments (Fig. 5) is a result of an unfavorable increase in the free energy at higher temperatures due to a positive change in ΔC_P. This principle explains well, why K^+ leak in MscS mutants with hydrophilic substitutions in the pore, such as A98S, is apparently heat sensitive (PTD phenotype). However, the change in ΔC_P cannot explain sufficiently the cold sensitivity (the NTD phenotype) of the mutants with hydrophilic substitutions (*e.g.* V65D and G76D) at the peripheral crevices unless the model incorporates allosteric coupling. In this model polar\charged residues introduced to the mutants are preferentially water exposed at higher temperatures. This phenomenon is either positively or negatively coupled to K^+ leak

Table 1
Localization of the NTD and PTD mutants on the MscS structure.

Mutation	Pore[a]	TM1/2-TM3a[b]	TM3b-ß-domain[c]	TM1/2-TM3b[d]	GOF[e]	LOF[f]	NTD[g]	PTD[h]
V65D		•				•	•	
G76D		•					•	
L82E		•			•			•
A94D	•						•	
I97N	•				•		•	
A98S	•				•			•
V99E		•			•			•
N117K			•				•	
L118P/Q				•			•	
A120D			•		•		•	
G121D			•			•		•
G168D			•				•	

[a] Localization at the pore facing side of TM3a.
[b] Localization at the crevices-facing side of TM2 or TM3a.
[c] Localization at the interface between TM3b and the ß-domain.
[d] Localization at the interface between TM1/2 and TM3b.
[e] Gain-of-function phenotype according to [11].
[f] Loss-of-function phenotype according to [11].
[g] Negative temperature dependence, Fig. 2.
[h] Positive temperature dependence, Fig. 2.

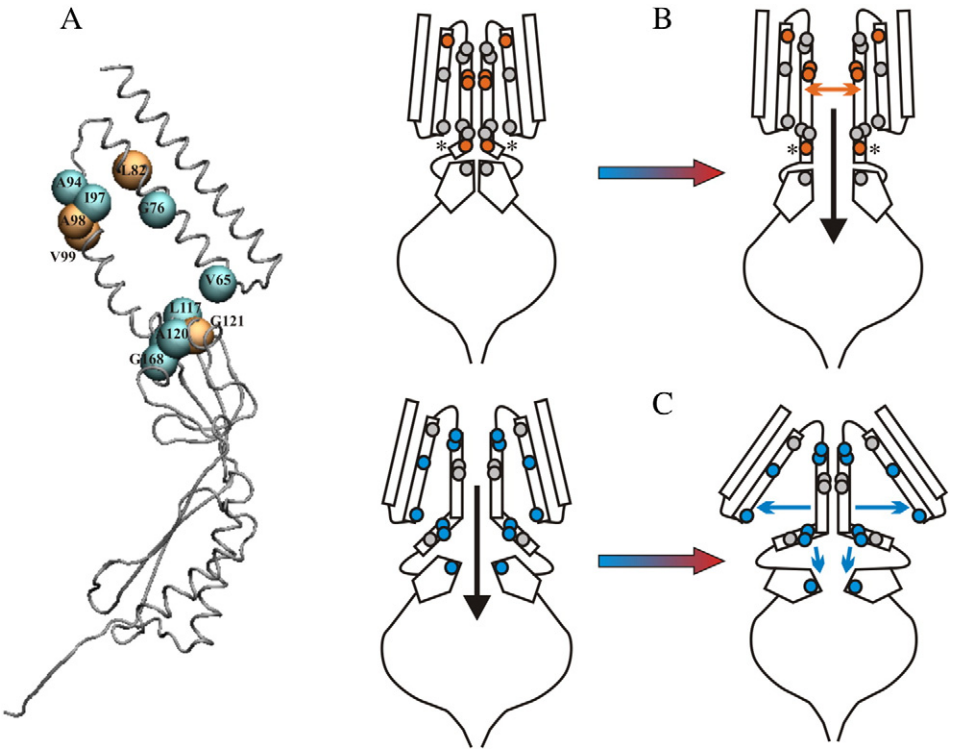

Fig. 6. A simplified model of the temperature dependent K^+-leak in the MscS mutants. A. The structure of a single subunit of MscS in an inactivated state (PDB ID: 2OAU). The positions of the amino acids, whose substitutions result in the PTD or NTD phenotypes are marked by the orange or blue balls, respectively. B. A cartoon depicting an increase of channel K^+ conductance in response to the rise in temperature (PTD phenotype) observed for the mutations L82E, A98S, V99E and G121D (orange balls). These mutations result in an excessive hydration of the pore vestibule, which is positively coupled to the K^+ leak. At low temperature the mutant channels reside in a closed-like state (left). Increase of temperature (arrow) results in the transition to an open-like state (right). The G121D mutation (marked by asterisk) removes a kink preferred in the closed conformation of the pore helix, what shifts the equilibrium towards the open-like state (see [10]). C. A cartoon depicting a decrease of channel K^+ conductance in response to the rise in temperature (NTD phenotype) observed for the mutations V65D, G76D, A94D, I97N, N117K, A120D and G168D (blue balls). These mutations result in a modest separation of the peripheral crevices, or the ß-domain from the pore helix, what allows for the K^+ leak at low temperatures (left). An increase of temperature (arrow) results in a large conformational change and the pore collapses similarly as during the MscS inactivation (right).

through the mutant channel pore underlying observed PTD and NTD phenotypes, respectively (Fig. 6). In general, MscS mutants provide a simple model for thermal gating, in which both: a change in heat capacity and an allosteric coupling modulate thermal response.

5. Conclusions

In this study, using MscS, a mechanosensitive channel from *E. coli*, we demonstrate the first direct experimental confirmation of the allosteric model of opposite temperature dependence of thermosensitive channels, which was provided to explain the opposite thermal dependence of TRP channels. From now on, MscS could be considered a model protein not only for mechanosensation but also for thermosensation.

Conflict of interest statement

The authors declare no conflicts of interest regarding paper entitled "Negative and positive temperature dependence of potassium leak in MscS mutants: Implications for understanding thermosensitive channels" by Piotr Koprowski, Malgorzata A. Sliwinska, Andrzej Kubalski.

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