Studies of allosteric coupling in KcsA using a constitutively open mutant

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Abstract

Transmembrane allosteric coupling in C-type inactivation of a potassium channel is analyzed using NMR. Activation of KcsA is initiated by a decrease in pH on the intracellular side of the channel. Numerous studies suggest that the selectivity filter is subsequently allosterically affected by opening and that a conformational switch and loss of affinity for potassium ions is the central step of C-type inactivation. We tested this hypothesis using an open pH gate KcsA mutant (H25R/E118A) which is constitutively active and lacks pH-dependent behavior of the activation gate. We use Solid State NMR measurements of this open pH gate mutant at neutral pH in hydrated bilayers to probe the potassium ion concentration dependence. The potassium ion binding affinity of the selectivity filter is 81 mM, about 4 orders of magnitude weaker than for wild type KcsA at the same pH, but similar to the value for wild type KcsA when activated at low pH. These results underscore the fact that ion affinity at the selectivity function is very much weaker in the open, activated state as compared to the closed, deactivated resting state, and confirm the role of allosteric coupling in the delayed inactivation mechanism. The protonation state of an important glutamate residue (E120) in the pH sensor of the activation gate changes as a function of potassium concentration, indicating that this mutant also exhibits transmembrane allosteric coupling. .

Introduction

Potassium channels selectively permit potassium ions to flow across biological membranes upon activation by an external stimulus such as voltage or nucleotide binding. Potassium channels typically contain a selectivity filter, which selects for potassium over other ambient ions with high affinity, as well as an activation 'gate' that opens the channel and initiates the flow of potassium. In many potassium channels, however, the channel spontaneously stops conduction even when potassium ions and activation stimuli are still present [9, 25]. This process typically occurs on a millisecond timescale after activation and is termed 'C-type inactivation'. Inactivation has slow kinetics and is highly modulated by permeant ions and pore blockers [45]. Understanding the structural basis of C-type inactivation is crucial to depict the regulation and drug response mechanism of biomedically important channels [1, 15].

KcsA is a prototypical bacterial potassium channel which is pH-activated. The pH 'gate' is located at the intracellular face of the channel, formed by a group of key ionizable residues including H25, E118 and E120 [7, 13, 18]. Like its more complex eukaryotic homologs, KcsA undergoes inactivation on the millisecond timescale after activation by a drop of pH [7, 19]. In previous studies, our and other groups

hypothesized that the site of inactivation is in the selectivity filter, where potassium ions are bound and conducted through the membrane [2, 9, 23, 40]. This mechanism is termed activation-coupled inactivation, i.e., and proposes that an allosteric coupling between the selectivity filter and activation gate (like the pH gate in KcsA) lead to a decrease in potassium affinity at the selectivity filter after activation. A direct measurement of the potassium ion affinity at the selectivity filter by solid state nuclear magnetic resonance (SSNMR) was carried out on KcsA embedded in an authentic lipid environment [42]. The potassium ion affinity changed from $K_{apparent} = 14 \pm 1$ mM at pH 3.5 to 4 ± 1 μM at pH 7.5. If the channel is open or activated at low pH, the potassium affinity is reduced at the selectivity filter, essentially leading to a conformational change at the selectivity filter, wherein the channel loses its grip on the ion, which in turn results in loss of potassium ion flow through the channel. This is consistent with findings from electrophysiology that increasing extracellular potassium ion concentration decreases the rate of inactivation [2, 40].

To further investigate allosteric coupling between the pH gate and the selectivity filter, we aim to characterize a mutation which renders the channel constitutively open at a neutral pH (a deactivation pH) also leads to a lower affinity at the selectivity filter. In the mutant H25R/E118A/E120A, the pH gate remains open from pH 4 to 9 [36]. This result is consistent with the finding by solution NMR data showing H25 is a pH sensor [34]. Subsequently, the minimal number of amino acids mutated to open the pH gate was found to be H25R and E118A [29]; we characterize this mutant to test whether this open state of the channel also lacks affinity for the potassium ions at the selectivity filter.

Materials and Methods

Expression and Purification of ^{13}C, ^{15}N-labeled open pH gate KcsA

The open pH gate H25R E118A KcsA was expressed with a plasmid previously prepared by the Crina Nimigean's laboratory. The protein expression and purification protocol were based on our former work with minor modifications [4]. 4 μl of the open pH gate KcsA plasmids was added into 50 μl JM83 competent cells in a culture tube. The mixture was incubated on ice for 30 minutes, then heat-shocked for 70 seconds on a 42 ^{o}C water bath and incubated on ice for another 3 minutes. 1 mL of S.O.C recovery medium (Invitrogen) was added, and the cells were incubated at 37 ^{o}C for 1 hour. The transformed cells were plated up to Luria broth (LB) agar plates containing 100 μg/ml ampicillin and incubated overnight at 37 ^{o}C. Single colonies of the transformed cells were picked and transferred to LB precultures with 100 μg/ml ampicillin. The preculture was incubated at 37 ^{o}C, 250 rpm, until the OD600 reached 1.0. The precultures were transferred into 4 $\times$ 1L LB. Once the OD600 reached 0.9, the cells were harvested via centrifugation at 4 ^{o}C, 8000 rpm for 15 minutes and resuspended in 1 L M9 medium with 3.0 g ^{13}C-labeled D-glucose, 0.5 g $^{15}NH_4Cl$. The cells were incubated at 37 ^{o}C for 1 hour to recover, and then protein expression was induced with anhydrotetracycline (Sigma), and incubated at 20 ^{o}C, 330 rpm overnight. The induced cells were harvested via centrifugation, resuspended in equilibrium buffer (100 mM KCl, 50 mM Tris, 2 mM decyl maltoside (DM), pH 7.5) and lysed by French Press. The cell membranes were extracted with 30 mM DM at 4 ^{o}C overnight. The unlysed cells and membranes were pelleted via centrifugation at 4 ^{o}C at 15000 rpm for 1 hour. The protein was purified by nickel affinity column and eluted with 200 mM Imidazole. Imidazole concentration was reduced by dialysis against the equilibrium buffer, and the protein solution was concentrated for reconstitution.

9:1 w/w DOPE and DOPS (Avanti) were mixed in test tube, the chloroform was evaporated by nitrogen gas flow, and the lipids was resuspended into equilibrium buffer at concentration of 10 mg/ml. The ^{13}C,^{15}N-labeled open pH mutant KcsA was reconstituted into DOPE/DOPS liposome at 1:1 protein to liposome weight ratio. The mixture solution was dialyzed against a solution of 50 mM Tris, x mM KCl , (100-x) mM NaCl (to compensate ionic strength, except for the 150 mM KCl sample) at pH 7.5 overnight for three times under room temperature. The proteoliposome pellets were harvested, then freeze-thawed at – 80 ^{o}C and room temperature for three times to remove bulky water. The final sample was reduced to a volume of around 30 μl by doing freeze-thaw cycle and packed into a 3.2 mm Bruker rotor, which corresponds to 10 mg mutant KcsA.

Solid-state nuclear magnetic resonance and data analysis

Magic-angle-spinning (MAS) solid-state NMR spectra were measured on Bruker 750 MHz (17.6 T) and 900 MHz (21.1 T) Avance spectrometer in New York Structural Biology Center (NYSBC). The MAS rate was set to 14 kHz and the variable temperature apparatus was set to 264 K. Typical radiofrequency (rf) field strengths were 93-109 kHz for 1H and 50-62.5 kHz for ^{13}C. ^{13}C chemical shifts were referenced externally to the downfield adamantane CH2 resonance chemical shift at 40.48 ppm [4]. 2D ^{13}C-^{13}C Dipolar Assisted Rotational Resonance (DARR) experiments were measured to obtain ^{13}C chemical shifts [26, 33]. The DARR mixing time was set at 15 ms. SPINAL64 decoupling on the 1H channel was set at 85-90 kHz during acquisition [32].

We calculated the population of the bound and apo states by integrating according cross peaks as previously did [42], the population of bound and apo state of marker peaks T75 CB-CA, T74 CB-CA, T75 AB-CG and V76 CB-CB were quantified by the intensity of the bound and apo state cross peaks by a sum over box method in sparky [16]. The spectra were processed in Sparky : peaks were picked according to former assignment [16, 41], and the integral (I) of the 2D peaks were integrated using a sum over box method, so that the bound population =

$$\frac{I_{bound}}{I_{bound} + I_{apo}} \times 100\%$$

The normalized bound ratios of the protein under different K^+ were calculated by averaging the bound ratio of the marker peaks. Data were plotted and fitted to a Hill method in OriginPro, and the function of the Hill method was:

$$\theta = \frac{x^n}{x^n + {k_d}^n}$$

In the formula, n denoted Hill coefficient. The average bound population increase from 0% at 10 mM [K^+] to 97% at 150 mM [K^+]. In the range of 50 mM to 100 mM [K^+], the major conformational transition takes place.

Electrophysiology

Freshly prepared T74S/H25R/E118A KcsA was prepared using former protocols and purified with a gravity Nickel column and desalting column on FPLC. 5 mg DOPE:DOPG 3:1 liposome were prepared: lipids in chloroform were dried under nitrogen gas, and pentane was added to get rid of all the organic solvents; dry lipids were resuspended by sonication in 1 ml HEPES swelling buffer while slowly adding CHAPS detergent to clear, in total 30mg CHAPS were added. 2.5 ug

T74S/H25R/E118A KcsA was added into the liposome, and the mixture were incubated under room temperature for 30 mins. Bio-beads column was used to get rid of the detergent, and the proteoliposomes were eluted at 8 ml and frozen in liquid nitrogen.

Electrophysiology experiments were carried out with a partition diameter of 100 μm. The lower chamber bath pH was at 4.0 (succinic acid buffer) and the upper chamber pH was at 7.0 (HEPES buffer, 100 mM K^+). Data were collected in Clampex and processed in Clampfit.

Results

Mutation at the pH gate does not directly change the conductive/collapsed conformation at the selectivity filter

NMR chemical shifts are precise indicators for protein structural and conformational changes. We conducted solid-state NMR experiments to investigate the structure of H25R/E118A mutant in the lipid bilayer environment (DOPE:DOPS = 9:1 w/w) with 1:1 w/w protein to lipid ratio, analogously to our prior studies [42]. As seen in Figures S1 and S2, the protein is well folded and displays high-quality NMR spectra at both the low (3.5) and high (7.5) pH where most markers are similar to the wild type. This confirms that H25R/E118A mutant is well folded in lipid bilayers. Moreover, the H25R/E118A mutant displays two conformations at the selectivity filter, analogously to the wild type. NMR chemical shifts report the conformational change that occurs during K^+ binding for the wild type, as has been extensively discussed in relation to the two major conductive and collapsed conformations seen in various crystal structures. Two sets of chemical shifts of the H25R/E118A mutant are observed for the selectivity filter in different [K^+], at both pH values. This indicates that H25R/E118A mutants do not directly cause a new conformation at the selectivity filter, which is consistent with that H25 and E118 have been shown to be proton binding sites.

Opening the pH gate significantly lowers potassium affinity at the selectivity filter at neutral pH

To probe allosteric coupling between the pH gate and the selectivity filter, we determined the potassium affinity of the open pH gate mutant H25R/E118A KcsA at neutral pH. As discussed above, E118&H25R mutant displays the K^+-bound (i.e. conductive) and K^+-apo (i.e. collapsed or inactivated) conformation. We used residues previously shown to have characteristic chemical shifts in the potassium binding site including T75 (CA, CB, CG), T74 (CA ,CB, CG), V76 (CA, CG) to assign the apo and K^+ bound population. The detailed cross peaks of marker peaks near the selectivity filter in 2D ^{13}C-^{13}C DARR spectra under 10, 50, 75, 80, 85, 100, 150 mM [K^+] were shown in Figure 1. (a-c). At intermediate [K^+], both the apo and bound peaks have significant intensities, indicating that the equilibrium of open and collapsed states of the open pH gate KcsA mutant is near these conditions. Also, no intermediate NMR lines are present at these conditions, indicating the conformations are in the slow exchange regime, characteristic of a bimodal conformational distribution, which is consistent with previous measurements for wild type KcsA, indicating that these mutations at the pH gate do not have a substantial change to the dynamics of the structural transition at the selectivity filter [42].

The NMR spectra showed that the KcsA channels were in the activated and inactivated states, and bound ratio against [K^+] could be fit to a Hill model. From the model fitting $K_{apparent}$ was extracted [42]. When Vmax is set to 1 and Hill coefficient is set free, the $K_{apparent}$ of H25R/E118A KcsA was best fit to 81 $\pm$ 1 mM in contrast

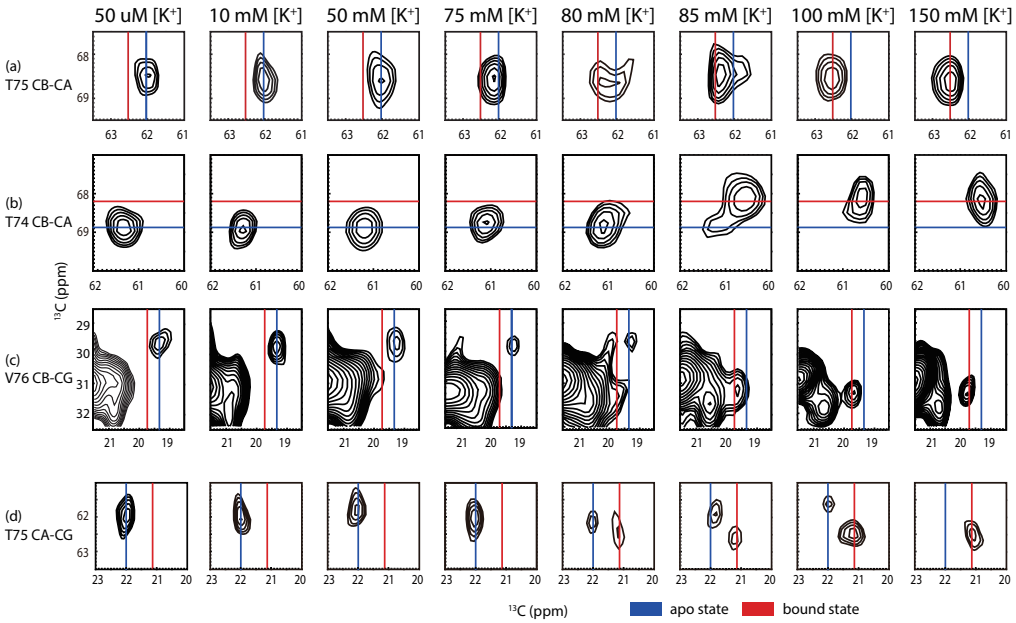

Figure 1. [K^+] dependence of the intensities of the apo and bound state cross peaks of marker peaks near the selectivity filter in 2D ^{13}C-^{13}C (a-d) correlation spectra of H25R/E118A KcsA at pH 7.5. (a) T75 CB-CA. (b) T74 CB-CA. (c) V75 CB-CG. (d) T75 CA-CG. (a-d) show that the marker peaks shift from apo state in low [K^+] to bound state in high [K^+]. The contour level of the spectrum was set at 5 times noise level. Marker peaks could be integrated to calculate the population ratio of apo and bound states.

with 4 ± 1 μM for wild type KcsA at pH 7.5 and 14 ± 1 mM for wild type KcsA at pH 3.5 (Figure 2). Details of the Hill fit were shown in Table. S1. An interesting observation from the titration curve for the H25R&E118A mutant is that the transition from the bound to the unbound state is very steep comparing to those for sample at pH7.5 and pH 3.5. We interpret this as an increasing in the cooperativity during the potassium ion binding at the selectivity filter. However, the system is complicated in terms of proton binding and potassium binding and the coupling between them, which prevent us from drawing a clean conclusion on this observation.

H25R/E118A mutant remains similar potassium affinity under low pH

To test if application of low pH could change the potassium affinity of the mutant ,We recorded solid state NMR spectrum at low pH. The bound ratio of mutant at 80 mM [K^+] is around 40%, which showed that the $K_{apparent}$ of the mutant at low pH was very close to the value at neutral pH (Figure S3). The little affinity difference confirms that after the pH gate is open in the H25R/E118A mutant, lowering pH does not further perturb the apo and bound population at the selectivity filter, indicating that these two perturbations affect the thermodynamics at the selectivity filter through similar coupling effect between the pH gate and the selectivity filter.

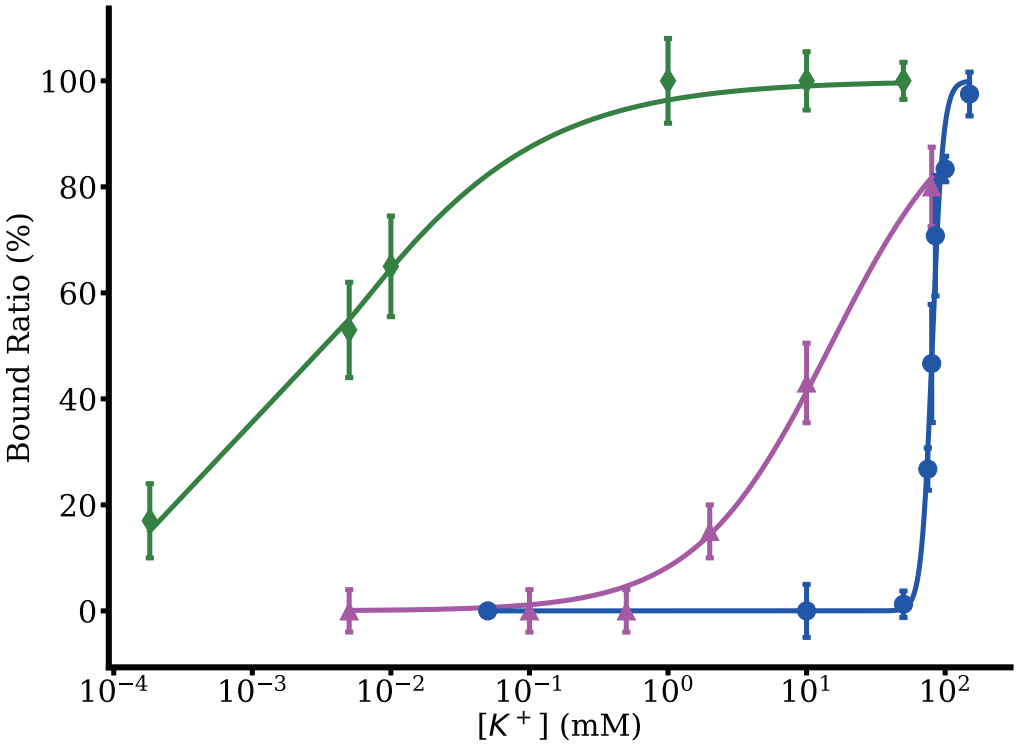

Figure 2. The bound ratio is plotted against $[K^+]$ to make the titration graph. The H25R/E118A (blue) is compared with our former data of wild type pH 7.5 (green) and wild type pH 3.5 (magenta). $K_{apparent}$ of the open pH gate mutant was calculated to be 81 ± 1 mM by fitting the data to a Hill binding model compared with 4 ± 1 μM for wild type at pH 7.5 and 14 ± 1 mM for wild type at pH 3.5 [42]. Our data show that keeping the pH gate open with H25R/E118A mutant further shift the K^+ affinity to a lower level.

pH gate is coupled

Allosteric coupling between the selectivity filter and the pH gate was demonstrated in previous studies [8, 10, 20, 21, 30, 35, 42]. Namely, not only do protonation and opening cause ion loss at the selectivity filter, but also protonation of the pH gate residues E118 and E120 can be caused by removal of ion and selectivity filter pinching at neutral pH. In prior spectra the peaks for E118 and E120 are congested and unresolved [41, 42]. In the H25R/E118A mutant, we monitored the spectra of E120 during the K^+ titration process, as shown in Figure 3. The E120 CG-CD peak indicated that this residue is protonated at low $[K^+]$,and deprotonated at high $[K^+]$ (Figure 3a). The intensity of the protonated peak of E120 CG-CD decrease but the intensity of the deprotonated peak increased with increasing $[K^+]$ (Figure 3b). At 50 μM $[K^+]$, E120 CG-CD appears to be essentially fully protonated, while at 80 mM $[K^+]$ the E120 CG-CD appears to be essentially fully deprotonated. These results show that in the H25R/E118A KcsA mutant, the pH gate and selectivity gate are coupled allosterically, and analogously to the wild type KcsA result [41].

Electrophysiology of the H25R/E118A mutant

It was previously shown that although the H25R/E118A mutant is open it does not conduct under standard electrophysiology conditions (pH 7.0, 100 mM $[K^+]$), because it,

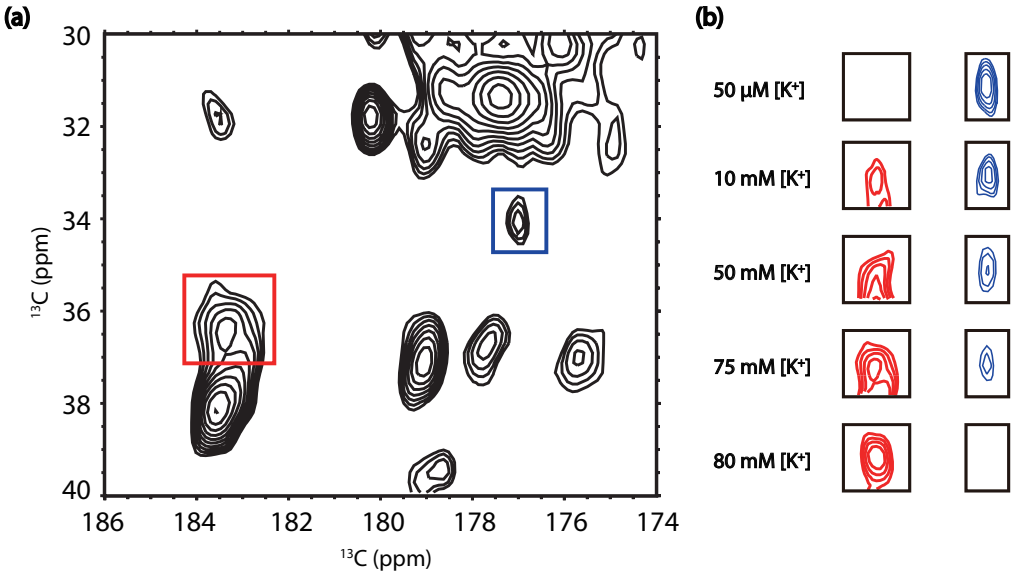

Figure 3. The effect of $[K^+]$ on E120 residue in the pH gate of the H25R/E118A KcsA mutant. 2D ^{13}C-^{13}C correlation spectrum of open pH gate KcsA at 75 mM $[K^+]$ is shown in (a). The peaks in the red and blue rectangles are the E120 CG-CD peaks in deprotonated and protonated state respectively. The protonated and deprotonated state change of the E120 peak as a function of $[K^+]$ is shown in (b). Protonated and deprotonated E120 CG-CD peaks at 50 μM, 10, 50, 75 and 80 mM are shown. At pH 7.5, E120 is protonated at low $[K^+]$, while at and above 80 mM $[K^+]$, E120 is completely deprotonated. In samples with $[K^+]$ larger than 80 mM, the E120 CG-CD peaks are all in the deprotonated state. This protonation state change provides clear evidence for pKa change of the glutamic acid residue in the pH gate.

like the wild type, inactivates when open. Inactivation is suppressed by preparing the triple mutant E71A/ H25R/E118A [30].

We recently identified several participants in allosteric coupling in KcsA using NMR [43] and the residues identified by NMR are in good agreement with other molecular dynamics and electrophysiology studies [22, 43]. These studies indicate that T74 is a key allosteric participant and a mutant T74S exhibits a strong reduction in allosteric coupling in KcsA. The Triple mutant E71A/H25R/E118A recovers essentially complete activity [29], and T74S/H25R/E118A mutant recovers about 30% of activity confirming as previously reported that T74S strongly (but perhaps not completely) reduces allosteric coupling and inactivation, and the pH gate mutations render the channel constitutively open (Figure S4). The difference between E71A and T74S in this context may be because they operate through very different mechanisms, where T74S is apparently right on the allosteric path, and E71Ais thought to be involved in stabilizing the selectivity filter. Evidently, mutations in the pH gate causes subtle differences in the selectivity filter as compared to the protonated wild type, highlighting the differences between the two inactivationless mutations. We speculate that, lacking the restraint of the hydrogen bonding network present in the wild type, the mutant may open to a different degree and thereby encourage inactivation.

Discussion

We used the open pH gate KcsA mutant H25R/E118A to study allosteric coupling in the inactivation process. Tetrametric KcsA was reconstituted in lipid bilayers and structural fingerprints were deciphered by SSNMR experiments. C-type inactivation develops as a delayed response when the channel is protonated at the pH sensor, and opened to for activated state, the explanation appears to be an allosteric coupling that results in a conformationally collapsed selectivity filter [11, 44]. In this study, we performed SSNMR experiments on open pH gate KcsA samples at different potassium concentrations, and offer support for this hypothesis outlined above. Specific marker peaks in the selectivity filter were used to determine the percentage of bound population in the inactivation process. The titration curve indicates that the potassium ion affinity of the KcsA mutant is 81 mM. Also, a pKa change of E120 residue at the pH gate upon ion removal showed that the pH gate was under strong allosteric coupling to the selectivity filter.

Because of C-type inactivation, potassium channels in the activated state spontaneously undergo inactivation and are difficult to capture for structural characterization under mild conditions [11]. Efforts to trap the two states of the C-type inactivation process have often lowering the pH [3, 5, 6, 20, 30, 37, 41]. Although lowering the pH seems to be an intuitively correct method to trap the activated and the inactivated state, we had concerns about nonspecific effects of low pH (on both lipids and protein). At physiological condition, lipids are critical to sustain the function of KcsA by stabilizing specific conformation of it [14, 27, 31, 39]. Performing measurements on a KcsA mutant with a constitutively open pH gate at physiological pH values effectively eliminates this concern. Different constitutively open KcsA mutants have been designed and characterized in previous studies, however, we select H25R/E118A to study because it has the lowest number of mutations which least interferes with the overall structure and function, meanwhile having the highest open probability at neutral pH [12, 17, 30, 37]. The four orders of magnitude reduction of potassium affinity at the selectivity filter comparing H25R/E118A to wild type KcsA at neutral pH (81 mM vs. 6μM) confirms that the large affinity change observed in the wild type KcsA is indeed due to allosteric control of ion affinity and activity (rather than nonspecific pH effects).

Our titration study sheds additional light on the mechanism of allosteric coupling in the inactivation process. It confirms that even though opening the pH gate does not directly affect the K^+ bound and apo structures of the selectivity filter, opening the pH gate does significantly affect the populations of the two conformations, and biases the system towards the apo conformation. Since the pH gate and the selectivity filter are almost 30 Å from each other, such a dramatic effect is due to an allosteric coupling. We note that experimental results from isothermal titration calorimetry (ITC) study on a similar mutant H25R/E118A/E120A with truncated C-terminus in a detergent environment show that the affinity for K^+ at pH 8 is 0.13 mM, close to the apparent potassium affinity value 0.15 mM for wild type KcsA in their measurement [24] . We attribute this inconsistency potentially to the usage of detergents in the experiments, which could potentially affect the structure and functions of membrane protein, as shown in study of KcsA and many other systems [9, 28]. Truncation of the C-terminus certainly changes the thermodynamics properties of the channel; in electrophysiology experiments, KcsA with truncated C-terminus showed a more rapid and complete inactivation [11, 38]. Solid state NMR measurements of full length KcsA absence of detergent logically better mimic the properties of the channel under physiological conditions.

The conformation change during inactivation, and the channels affinity for potassium ion were characterized using NMR chemical shifts as a function of potassium ion concentrations. The potassium ion affinity of the open pH mutant is in the same order of magnitude as the affinity of wild type KcsA channel under acidic conditions.

This result further confirms the hypothesis that allostery and loss of ions in the selectivity filter underlie the activation coupled inactivation mechanism. The pKa of the E120 at the intracellular pH gate was also shown to be influenced by potassium ion binding at the selectivity filter, providing additional evidence for an allosteric coupling network in KcsA.

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