Recovery from slow inactivation in $K^+$ channels is controlled by water molecules

Jared Ostmeyer, Sudha Chakrapani, Albert C. Pan, Eduardo Perozo & Benoît Roux

Application of a specific stimulus opens the intracellular gate of a $K^+$ channel (activation), yielding a transient period of ion conduction until the selectivity filter spontaneously undergoes a conformational change towards a non-conductive state (inactivation). Removal of the stimulus closes the gate and allows the selectivity filter to interconvert back to its conductive conformation (recovery). Given that the structural differences between the conductive and inactivated filter are very small, it is unclear why the recovery process can take up to several seconds. The bacterial $K^+$ channel KcsA from *Streptomyces lividans* can be used to help elucidate questions about channel inactivation and recovery at the atomic level. Although KcsA contains only a pore domain, without voltage-sensing machinery, it has the structural elements necessary for ion conduction, activation and inactivation. Here we reveal, by means of a series of long molecular dynamics simulations, how the selectivity filter is sterically locked in the inactive conformation by buried water molecules bound behind the selectivity filter. Potential of mean force calculations show how the recovery process is affected by the buried water molecules and the rebinding of an external $K^+$ ion. A kinetic model deduced from the simulations shows how releasing the buried water molecules can stretch the timescale of recovery to seconds. This leads to the prediction that reducing the occupancy of the buried water molecules by imposing a high osmotic stress should accelerate the rate of recovery, which was verified experimentally by measuring the recovery rate in the presence of a 2-molar sucrose concentration.

Available X-ray structures of the KcsA channel provide an atomic view of the four most important functional states in which the intracellular gate is either closed or open, and the selectivity filter is either conductive or inactivated. These are (in terms of their Protein Data Bank entries): the closed-conductive (1K4C), open-conductive (3FB7), open-inactivated (3F5W), and the closed-inactivated (1K4D) states (Fig. 1a). When the filter is in the conductive state (1K4C and 3FB7), it is occupied by several $K^+$ ions arranged in single file over five binding sites (S0 to S4); ion occupancy is considerably reduced when the filter is in the non-conductive inactivated state (1K4D and 3F5W) because it is ‘pinched’ at the central glycine residue of the signature sequence TVGTYGD. The conductive conformation of the filter is virtually unchanged when the gate is closed (1K4C) or open (3FB7). The pinched non-conductive conformation of the filter is similar in the open-inactivated conformation (3F5W) and in the closed structure (1K4D) crystallized at low $K^+$ concentration, indicating that the latter provides a realistic representation of the KcsA channel in the closed-inactivated state.

Although available X-ray crystal structures reveal the conformations of the functional states, they do not explain the experimentally observed timescales. Recovery from inactivation is an extremely slow, ion-dependent process. Macroscopic current measurements show that the KcsA channel inactivates within 1–3 s and that recovery takes place in 5–20 s (Fig. 1b, c), depending on the external $K^+$ concentration. However, the structural difference between the conductive and non-conductive filter is less than ~1 Å root mean square deviation (r.m.s.d.), barely larger than the thermal fluctuations of proteins under ambient conditions (Fig. 1d). According to a naive Eyring rate theory argument, the long timescale corresponds to an activation free energy barrier of about 15–20 kcal mol$^{-1}$, a value that is difficult to reconcile with the very slight structural difference between the conductive and inactive filter (Supplementary Fig. 1). Explaining the molecular origin of the extraordinarily long timescale of the recovery process is an unresolved issue.

To identify the rate limiting step opposing recovery, a molecular dynamics simulation of KcsA with the non-conductive filter and the closed gate was carried out in the presence of a high K$^+$ concentration. Although these conditions were designed to favour a spontaneous transition of the filter towards the conductive state, the filter remained...
in the non-conductive state during the 17-μs-long simulation (Fig. 2a). Throughout the simulation, the selectivity filter stayed near the crystallographic conformation 1K4D and no ion translocation event was observed (Supplementary Fig. 2). The stability of the non-conductive filter over this long timescale indicates that the simulation captured the rate-limiting step opposing a rapid recovery of the filter.

Examination of the 17 μs trajectory led us to focus on three buried water molecules in an ~8-Å-long cavity located behind the selectivity filter of each subunit. Whereas water molecules bound near the protein surface are expected to undergo rapid dynamical fluctuations and exchange easily with the bulk, the buried waters maintained distinct positions and orientations throughout the entire simulation. The buried waters were stabilized by a network of water–water and water–protein hydrogen bonds (Fig. 2b), displaying long residence times (Supplementary Table 1). The buried water molecules thereby become an integral part of the protein structure when the selectivity filter is in the pinched conformation. On average, a given subunit cavity is occupied by three buried water molecules ~99% of the time as each cavity releases its waters into the bulk no faster than 10–15 μs and refills in 80–100 ns (Supplementary Fig. 5). The Tyr 82 side chain acts as a lid, controlling access to the cavity. In the cavity, the buried water molecules remain near the location observed in the structure with the pinched non-conductive filter (1K4D), which is also consistent with nuclear magnetic resonance (NMR) data. The outermost water molecule is observed in the X-ray structure of the conductive filter (1K4C), whereas the two others are absent. Structural modelling shows that the latter would clash with the backbone Cα of the central glycine residue (Gly 77) if the filter adopted a conductive conformation (Fig. 2c). These results indicate that the presence of the buried water molecules bound behind the filter locks its conformation into the pinched non-conductive state and prevents a spontaneous transition towards the conductive state.

To demonstrate that these ‘inactivating water molecules’ prevent recovery, additional molecular dynamics simulations were carried out in which they were removed (Fig. 2d). Within a few nanoseconds, the filter made a transition towards the conductive conformation (1K4C). The recovery transition was accompanied by K+ ions translocating inward towards the filter from the extracellular side and the binding of water molecules behind the filter at the positions observed in 1K4C. While one K+ ion moved to the site S2 of the filter, another entered the site S0 from the extracellular bulk. These two K+ ions, combined with a third K+ ion in S4 that remained present at its initial location during the entire simulation, resulted in a ‘S0–S2–S4’ ion configuration in the filter. The involvement of K+ ions is expected, as the timescale of recovery increases at low extracellular K+ concentration. To clarify the role of the ions further, molecular dynamics simulations were carried out with no free K+ ions in solution. In all cases, the filter remained in the pinched non-conductive conformation, consistent with the experimental observation that low extracellular K+ inhibits recovery (Fig. 1c). In contrast, additional simulations in 1 M KCl showed that whether or not a K+ ion was initially bound in the site S4 or in the intracellular cavity of the channel, a K+ ion entered the filter from the extracellular side to bind to the site S2. Additional molecular dynamics experiments were carried out to determine the outcome from different intermediate starting conditions (Supplementary Table 2), indicating that the most likely pathway leading to recovery requires the absence of all three inactivating water from all four subunits to permit the binding of an external K+ ion to the filter.

To characterize the free energy landscape controlling the recovery process, we calculated the two dimensional potential of mean force (PMF) as a function of the pinched glycine Cα–Cα distance of the filter r and the position of the external K+ ion Z (Supplementary Fig. 6), with and without the inactivating water molecules (Fig. 3). These two states illustrate the coupling between the inactivating waters and the external K+ ion on the recovery process. When the inactivating water molecules are present (Fig. 3a), no significant free energy barrier is encountered to move a K+ ion from the bulk to the binding site S1 of the pinched filter pinned filter (snapshot i), but the subsequent transition of this external K+ to the binding sites S2 or S3 is energetically forbidden; the free energy landscape rises sharply, up to 30 kcal mol⁻¹ relative to the local minimum, as the K+ is brought deeper into the filter towards the site S2 along the Z coordinate. There is a large energy barrier because the non-conductive filter is too narrow for a K+ ion to fit through. The energy barrier to gradually open the filter to a conductive state is brought deeper into the filter towards the site S2 along the Z coordinate. There is a large energy barrier because the non-conductive filter is too narrow for a K+ ion to fit through. The energy barrier to gradually open the filter to a conductive conformation is also highly unfavourable, rising by ~25 kcal mol⁻¹ when an ion is in the site S1 (snapshot j). Opening the pinned filter by moving along the r coordinate is opposed by a large free energy barrier because the backbone of the filter clashes with the buried inactivating waters. The PMF with the inactivating waters removed is markedly different (Fig. 3b). Once a K+ ion reaches the site S1 (snapshot k), the recovery process becomes energetically downhill. A free energy basis guides the recovery process as the free energy decreases (snapshot l). The filter first makes a transition from the pinched to the conductive conformation, the ion in S1 then permeates down to the site S2 (snapshot m), ending in a conformation that is highly similar to the 1K4C structure. In the absence of inactivating waters, the channel will spontaneously and rapidly recover to a conductive conformation upon the

![Figure 2](image-url)
binding of a K\(^{+}\) ion from the extracellular solution. But the filter remains locked in the non-conductive state, even with an incoming external K\(^{+}\) ion, as long as the inactivating waters are present.

These computational experiments suggest that two factors control the conformational transition of the selectivity filter from the non-conductive pinched state to the conductive state: the release of the inactivating water molecules and the binding of an external K\(^{+}\) ion. The buried inactivating waters act as gatekeepers, locking the filter in the pinched non-conductive conformation; the binding of an external K\(^{+}\) ion acts as a catalyst for the final step; releasing the inactivating water molecules and the binding of an external K\(^{+}\) ion must then bind to the filter to catalyse the final step to recovery. Although these simulations suggest a plausible mechanistic scenario, a complete and spontaneous recovery from an inactivation event was not observed during these experiments. The recovery process, which occurs over a timescale of \(~10\) s, is longer than what can be achieved with state-of-the-art computing technologies. Despite these limitations, it is important to bridge the results from molecular dynamics experiments to the extremely long timescales of the recovery process observed in macroscopic current measurements. For this purpose, we rely on a kinetic model (Fig. 4a) based on the assumption that occupancy of the cavity of each of the four subunits by inactivating waters is independent and uncorrelated, and that recovery can proceed to its final stage through the binding of an external K\(^{+}\) ion only after all of the inactivating water molecules have been released. Although it is possible that recovery might occur very infrequently under different circumstances (Supplementary Table 2), these constraints capture the dominant mechanism of recovery. Occupancy changes take place with forward and backward rates of \(k_{f}\) and \(k_{b}\), followed by a final concerted step corresponding to the binding of an external K\(^{+}\) ion to the selectivity filter. By virtue of the stoichiometric constraint posed by the four subunits, the timescale of recovery predicted by this model is tens of seconds (Fig. 1c, dashed line) using microscopic rates that are consistent with the current molecular dynamics simulations (Fig. 4). Moreover, the model displays the correct sensitivity to the external concentration of K\(^{+}\), becoming slower at low concentration and reaching a plateau at high concentration.

This mechanism predicts that lowering the occupancy of the subunit cavities by inactivating water molecules should accelerate the rate of recovery from inactivation. This is consistent with the experimentally observed hydrostatic pressure dependence of recovery for the Shaker K\(^{+}\) channel\(^{14}\), which indicates that \(~8\) water molecules must be released from the inactivated state to return to the conductive filter. An alternative approach to vary the occupancy of the cavities is to apply stress from the external solution should accelerate the process of recovery; the stability of the inactivating waters in 2 M sucrose would decrease due to the high osmotic stress, which should lead to a more rapid time of recovery (Supplementary Fig. 7). To test this prediction, we measured the rate of recovery of wild-type KcsA channel in the presence of 5 mM of K\(^{+}\) and 2 M sucrose in the external solution (Fig. 4b).

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**Figure 3** Two-dimensional free energy landscape of the recovery process. The horizontal reaction coordinate \(r\) describes the width of the selectivity filter and is defined as the cross-subunit pinching distance between the Cz atoms of Gly 77. The vertical reaction coordinate \(z\) is the height of a K\(^{+}\) ion relative to the centre of mass of the selectivity filter. a, PMF calculated with inactivating water molecules present behind the selectivity filter. The pinched filter rests in a free energy minimum with a K\(^{+}\) ion in position S1 (snapshot i). The transition from a pinched to a conductive conformation (snapshot j) is the greatest free energy barrier relative to the local minimum, resulting in an unstable conformation of the conductive filter. b, PMF calculated with the inactivating water molecules absent. The pinched filter with a K\(^{+}\) ion in position S1 (snapshot k) recovers spontaneously, following the downhill slope of the free energy landscape. The filter recovers to a conductive conformation by moving first to an open conformation (snapshot l) before ions in the filter adopt a conductive configuration (snapshot m).

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**Figure 4** Impact of water molecules on recovery process. a, Kinetic scheme incorporating the main findings of the molecular dynamics simulations. The model was used to produce the simulated recovery times as a function of [K\(^{+}\)] shown in Fig. 1c (dashed line) using the rate constants 1/k\(_{f}\) = 11 µs, 1/k\(_{b}\) = 79 ns, 1/k\(_{m}\) = 8.3 ns \times (150 mM/[K\(^{+}\)])). When starting the kinetic model in the state with no inactivating water molecules (W), the channel reaches the active conductive state rather than the fully inactive state with a probability of \(~0.5\) at 50 mM of [K\(^{+}\)]. This probability rises to \(~0.7\) at 150 mM of [K\(^{+}\)]. b, c, Effect of external sucrose on the time course of recovery. b, Outward currents recorded with external 5K\(^{+}/145\) mM NMG and internal 150 mM K\(^{+}\) in the absence and presence of 2 M external sucrose. c, Fractional recovery averaged from eight patches plotted as a function of the inter-pulse interval. Error bars denote standard deviation.
absence of sucrose, the average recovery time is 17.06 ± 4.75 s, and in the presence of sucrose it decreases to 12.21 ± 3.73 s, consistent with our prediction (mean ± s.d.; Supplementary Fig. 7). Although the overall effect is small (the waters bound to one subunit are destabilized by only ~0.7 kBT, where kBT is Boltzman’s constant and T is the temperature), protein dynamics and the diffusion and binding of an external K⁺ ion to the selectivity filter that is critical to catalyse recovery are expected to considerably slow down in a viscous environment⁴⁸. The fact that the recovery process is accelerated in 2 M sucrose—despite a viscosity that is 30 times that of pure water—is strong evidence that the applied osmotic stress decreased the occupancy of the gatekeeper inactivating water molecules locking in the non-conductive state of the selectivity filter.

Our molecular dynamics simulations and macroscopic current measurements suggest that the selectivity filter of K⁺ channels functions as a ligand-gated pore through a built-in osmometer, where water molecules from the external solution are the ‘ligands’ responsible for the gating stimulus. It is unlikely that such a mechanism could have an important physiological role in higher organisms, as there are negligible variations in internal osmotic strength; however, it is possible that such a mechanism could regulate bacterial K⁺ channels, as micro-organisms are often exposed to widely varying external conditions. Examination of homology models of various K⁺ channels indicates that the subunit cavities are conserved structurally and in terms of the hydrogen bonding interactions that they could provide through the peptide backbone (Supplementary Fig. 3), indicating that the role of inactivating waters acting as gatekeepers of the pinched non-conductive filter is likely to be conserved in the K⁺ channel superfamily. The present analysis may provide a basis to understand the wide range of inactivation and recovery phenotypes arising from point mutations in the neighbourhood of the selectivity filter⁴⁴,⁷,⁹,¹⁷.

METHODS SUMMARY

Initial coordinates for the molecular dynamic simulations were taken from the crystal structures 1K4C and 1K4D. Crystallographic water molecules around the single subunit in each of the two crystal structures were placed around all four subunits in the tetramer. Residues were assigned their standard protonation state at pH 7.0, except for residue Glu 71, which was protonated. The channel was embedded in a bilayer of POPC lipids and solvated in 1 M KCl using the web service CHARMM-GUI (total number of atoms, 45,898). All-atom simulations were run using the CHARMM PARAM27 force field¹⁰ under constant NVT conditions at a temperature of 310 K. Molecular dynamic simulations were carried out on the special purpose computer Anton¹¹ on loan to the Pittsburgh Supercomputer Center (PSC). A total of 114 molecular dynamic simulations were carried out starting from different initial conditions with respect to the inactivating water molecules, for an aggregate total time of 29.5 μs of molecular dynamics.

Full Methods and any associated references are available in the online version of the paper.

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Author Contributions J.O. carried out all the final molecular dynamics simulations and 2D-PMF calculations and the computational analysis; A.C.P. initiated the molecular dynamics simulations and the 2D-PMF calculations; B.R. designed and simulated the kinetic models; S.C. and E.P. planned, carried out and analysed the experiments; B.R. conceived and supervised the entire project. All authors contributed to writing the manuscript.

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METHODS

Initial coordinates for the molecular dynamics simulations were taken from the crystal structures 1K4C and 1K4D. Crystalllographic waters around the single subunit in each of the two crystal structures were placed around all four subunits in the tetramer. Residues were assigned their standard protonation state at pH 7.0, except for residue Glu71, which was protonated. The channel was embedded in a bilayer of POPC lipids and solvated in 1 M KCl using the web service CHARMM-GUI (total number of atoms, 45,898). All-atom simulations were run using the CHARMM PARAM27 force field under constant NVT conditions at a temperature of 310 K. Molecular dynamics simulations were carried out on the special purpose computer Anton on loan to the Pittsburgh Supercomputer Center (PSC). A total of 114 molecular dynamics simulations were carried out starting from different initial conditions with respect to the inactivating water molecules, for an aggregate total time of 29.5 µs of molecular dynamics (Supplementary Table 2).

The two-dimensional PMFs (Fig. 3) with respect to the two coordinates r and z (Supplementary Fig. 6) were calculated using NAMD 2.9 (ref. 21), which were carried out on the petaflop supercomputer Jaguar, located at the National Center for Computational Sciences (NCCS). The region of interest in the (r, z) space was covered by a grid of equally spaced umbrella sampling (US) windows. To improve the statistical sampling, the US calculations were performed using Hamiltonian Replica Exchange MD (US/H-REMD) simulations (also called US window swapping22). Initial coordinates for the US windows were taken from the unbiased trajectories carried out on Anton. Initial coordinates for the missing windows were obtained by gradually dragging neighbouring windows along the reaction coordinates to the centre of each of the missing windows. Initial coordinates for the first PMF came from the 17-µs-long unbiased molecular dynamics simulation of the stable, pinched filter (Fig. 2a). Windows taken from this trajectory already contained three inactivating water molecules lodged behind the selectivity filter in the cavities of each of the four subunits. A series of flat-bottom harmonic distance restraints were added between channel atoms and the water oxygen of the inactivating waters to ensure that they remained present behind the filter. The flat portion of the restraints spanned 8 Å above the pore helix of the channel—providing the water molecules freedom to move around a region of space purposefully designed to be larger than the overall size of the cavity. The initial coordinates for the second PMF came from the molecular dynamics trajectory of the recovery process (Fig. 2d), providing windows spanning the conformational transition from the pinched to the conductive filter. Windows taken from this trajectory lacked all inactivating water molecules behind the filter. To maintain the system in this occupancy state during the US/H-REMD simulations, dummy atoms (no non-bonded interactions with the channel) positioned above the pore helix were introduced to repel any water molecule trying to enter into the cavities behind the filter from the bulk region. The first PMF comprised 137 windows whereas the second PMF consisted of 226 windows spanning the complete recovery process. All windows were equilibrated ≥0.5 ns before starting REMD. Exchange attempts were made every 1,000 steps (or 1 ps of simulation of time), and neighbouring windows were swapped if the Metropolis Monte Carlo exchange probability was satisfied. US/H-REMD simulations for the two PMFs were run for more than 1 ns. The total aggregate simulation time used to produce the two USH-REMD calculations is more than 800 ns. Windows were unbiased using the Weighted Histogram Analysis Method (WHAM)23,24, which only required that the umbrella sampling windows were generated according to Boltzmann statistics.

Electrophysiological measurements were made by patch-clamp recordings in channel-reconstituted liposomes prepared as described earlier25,26. Purified protein was reconstituted in a sootcin vesicles (in 1:100 (mass:mass) protein to lipid ratio) by dilution with 200 mM KCl and 10 mM MOPS buffer at pH 7.0. Residual detergent was further removed by incubation with biobeads (Bio-Rad Laboratories). Channel-incorporated liposome suspension was then centrifuved for 2 h at 100,000 g and the pellet was re-suspended in 60 µl of KCl/MOPS buffer. A drop of the proteoliposome was placed on a glass slide and dried overnight in a desicator at 4 °C. The sample was then rehydrated with 20 µl of buffer, which yielded giant liposomes. This preparation was suitable for patch-clamp recordings after ~2 h. Currents were recorded in 10 mM MOPS buffer with indicated salt concentration. For measurements under KCl gradient, N-methyl-D-glucamine was used to replace KCl in the pipette. Recording pipettes were pulled from thin-walled borosilicate glass and heat polished such that they had a bath resistance of 1–2 MΩ when filled with 200 mM KCl, 10 mM MOPS solution. All measurements in this study were conducted in the inside-out configuration of the patch clamp technique. Experiments were performed at room temperature (20–22 °C). Currents were elicited in response to pH jumps from 8.0 to 3.0 using an RCS-160 fast solution exchanger (Biology) fed by gravity. Macroscopic currents were sampled at 5 kHz using Axon 200-B patch-clamp amplifier. For each experiment in the absence or presence of sucrose, the fractional recovery was measured at different inter-pulse intervals. There are eight sets of experiment without sucrose, and eight with sucrose. For the two conditions, the average recovery curve and its standard deviation was determined from the eight different sets, and the time constant was determined from a single-exponential least-square fit. To determine if the recovery time constant in the absence and presence of sucrose is significantly different, we fitted each of the eight individual data sets (for the two conditions) by a single exponential and determined the recovery time constant for each experiment. The two sets of eight values were subjected to an unpaired Student’s t-test (degrees of freedom = 4), which yielded a P value of 0.03969.

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