The voltage-dependent gate in MthK potassium channels is located at the selectivity filter

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Understanding how ion channels open and close their pores is crucial for comprehending their physiological roles. We used intracellular quaternary ammonium blockers, electrophysiology and X-ray crystallography to locate the voltage-dependent gate in MthK potassium channels from Methanobacterium thermoautotrophicum. Blockers bind in an aqueous cavity between two putative gates: an intracellular gate and the selectivity filter. Thus, these blockers directly probe gate location—an intracellular gate will prevent binding when closed, whereas a selectivity filter gate will always allow binding. Kinetic analysis of tetrabutylammonium block of single MthK channels combined with X-ray crystallographic analysis of the pore with tetrabuty1 antimony unequivocally determined that the voltage-dependent gate, like the C-type inactivation gate in eukaryotic channels, is located at the selectivity filter. State-dependent binding kinetics suggest that MthK inactivation leads to conformational changes within the cavity and intracellular pore entrance.

Ion channel gating, the opening and closing of the pore is central to the regulation of ion movement across biological membranes. For some K+ channels, the primary activation gate is thought to reside at the intracellular end of the pore as a hydrophobic constriction of transmembrane helices, also called the bundle-crossing gate. This concept originated from groundbreaking channel block experiments using the giant squid axon and was later refined by numerous studies of voltage-gated K+ (Kv) channels. In particular, the primary ligand-controlled gate has been proposed to lie at the selectivity filter rather than at a cytoplasmic location of the voltage-dependent gate.

Kv channels also have a secondary gate, called the C-type inactivation gate, located within the selectivity filter at the extracellular end of the pore. Though many types of studies have converged on the selectivity filter model for C-type inactivation gating, only a subset of studies have investigated the gate location with a physical probe in order to assess the bundle-crossing gate. Specifically, quaternary ammonium blockers and N-type inactivation peptides block the pore cavity, located between the bundle-crossing and selectivity filter gates, in the inactivated state, and methanethiosulfonate reagents can react to cysteines inside the inactivated-state cavity. These studies indicate that C-type inactivation may involve conformational changes below the selectivity filter while keeping the activation gate open. Therefore, two distinct gates have been identified within K+ channel pores and may function in other K+ channel families.

Several studies of ligand-gated K+ channels have challenged the notion that related channel families share gating features with Kv channels. In particular, the primary ligand-controlled gate has been proposed to lie at the selectivity filter rather than at a cytoplasmic constriction. Further complications have arisen in the analysis of BK channels, which are gated by both ligand (Ca2+) and voltage. Despite the similarity between BK and Kv channels, several studies have suggested that the activation gate lies within the selectivity filter. Taken together, these studies raise questions about the origins and structural differences underlying these gating mechanisms.

The MthK channel is a model Ca2+-activated K+ channel related to BK channels but without voltage-sensor domains. Ca2+-dependent gating of MthK was proposed to occur at the intracellular end of the pore by the straightening of inner helices to form a bundle crossing, although MthK has only been crystallized in an open state. Additionally, at positive voltages MthK closes even in the presence of activating Ca2+, a gating process that has been shown to depend on extracellular ion composition similar to C-type inactivation in Kv channels and hence inferred to occur at the selectivity filter. At this time, however, the physical locations of the gates in MthK have not been directly probed either structurally or functionally.

In this study, we set out to conclusively determine the location of the voltage-dependent MthK gate by analyzing the state-dependent block of single-channel recordings. The use of quaternary ammonium blockers to analyze voltage-dependent gating can be ambiguous and conditional on the model because blocker binding is also voltage-dependent. In MthK channels, however, the voltage dependence of blocker affinity and gating occur over different voltage ranges.

At negative potentials, blockers couple to permeant K+ ions, which confer voltage dependence to the open-channel block. At positive potentials, open-channel block becomes voltage independent, which is critical in determining blocker kinetics during MthK gating and the location of the voltage-dependent gate.

We investigated the state-dependent microscopic block kinetics for tetrabutylammonium (TBA), tetrabuty1 ammonium (TPeA),

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benzyltributylammonium (bTBA) and N-(4-[benzoyl]benzyl)-N,N,N-
tributylammonium (bbTBA). We found that voltage gating did not
prevent channel block but modestly reduced the on-rate for all block-
ers. Therefore, the voltage-dependent (inactivation) gate is above
the blocker binding site at the selectivity filter, and the entrance to the pore
may be slightly constricted during inactivation. Interestingly, the off-
rates changed in a blocker-dependent manner during inactivation, likely
reflecting structural changes below the selectivity filter that accompany
voltage gating but do not prevent blocker access. Finally, an analysis of
X-ray diffraction data from a MthK pore in the presence of tetrabutyl
antimony (TB5b) indicated that the binding site is immediately below the
selectivity filter, as observed in KcsA structural data.8,37–39 State-
independent access of blockers to this site directly confirmed that the
MthK voltage gate is located at the selectivity filter.

RESULTS
We set out to directly identify the location of the MthK voltage gate
using quaternary ammonium probes that bind within the aqueous
cavity of K+ channels and block the current.3,26,39–41 If the binding site
is located beyond an ion channel gate, the channel has to open
for the blocker to bind, a process called gated access (Fig. 1a). This
corresponds to the intracellular-facing gate described for K+ channels.
In particular cases, the channel cavity may allow intracellular
gate closure with the blocker bound, thereby physically trapping it
inside (Fig. 1a).3,6,38 Alternatively, if the channel gate is at the select-
vicity filter, the blocker can bind to both the closed and open states
(Fig. 1b) and blocker binding is characterized by state-independent
access to the block site.

We studied state-dependent accessibility of the blocker during
voltage-dependent gating of MthK to determine the location of the
gate. Above 50 mV, voltage leads to a reduction in open probability
($P_o$) (Fig. 1c), as previously shown.35 Charged blockers, such as qua-
ternary ammonium ions, are known to block ion channels in a voltage-
dependent manner both during voltage-dependent gating3,26 and in
the absence of gating.5,62 In order to separate the two processes,
we first analyzed the blocker’s voltage dependence at voltages less than
50 mV, at which point there is no voltage gating (Fig. 1c).

Microscopic block of open MthK
We measured MthK block by TBA, bTBA and bbTBA (Fig. 2a) using
single-channel recordings in planar lipid bilayers under conditions
that maximized $P_o$.43 We first analyzed block of open MthK (−125 to
50 mV, Fig. 1c). Typical currents with and without blockers are shown
in Figure 2a. Excursions to the closed state in the control recording,
called flickers, are very brief and infrequent, becoming negligible at
positive voltages (Supplementary Fig. 1).33

![Image](https://example.com/image.png)

Figure 1 Block mechanism with two gate locations. (a,b) MthK pore cartoons illustrating gated access and blocker trapping (a) versus state-independent
access of blockers (b). Selectivity filter is red with two K+ ions bound (green). Inner-pore helices (gray) may form a bundle-crossing gate. (c) Mean
open probability ($P_o$) measurements for MthK in symmetric 200 mM K+ ($n = 3–8$, mean ± s.d., error bars smaller than symbols). Inactivation gating
(50–125 mV, red) is described by voltage-dependent transitions between one open (O) and one inactivated (I) state and was fit to a Boltzmann function
(red line, equation (1) with $P_o^{\text{max}} = 0.994 ± 0.004$, $z = 1.44 ± 0.02$ and $V_{1/2} = 103.3 ± 0.2$ mV (Supplementary Fig. 1a). The black line has no
theoretical meaning.

Addition of blockers decreased $P_o$ by increasing the time the channel spent in a nonconducting state. bbTBA blocked with the
highest affinity, followed by bTBA and TBA. This correlated with the
hydrophobicity increase caused by each additional benzyl group in
the blocker structure (Fig. 2a). A similar hydrophobicity-dependent
block has long been known for other K+ channels.3,40,41 The difference
in blocker affinities reflects a dramatic change in kinetics, with bbTBA
inducing longer closings than the other blockers (Fig. 2a).

To quantitatively analyze block kinetics, we used the QuB pro-
gram. Control recordings were fit with a two-state model, yielding
single-exponential fits to the open and closed dwell-time distributions
(Fig. 2b). Addition of the blockers introduces another closed com-
ponent, requiring a model with two nonconducting states (Scheme I,
Fig. 2b). Blocker on-rates were proportional to blocker concentration,
whereas off-rates were concentration independent, consistent with the
bimolecular association shown in Scheme I (Supplementary Fig. 2).
The dissociation constant of the blocker for the open state ($K_{d^{\text{open}}}$)
was calculated from these rate constants. We investigated next how
these parameters ($k_{\text{on}}, k_{\text{off}}$ and $K_{d^{\text{open}}}$) varied with voltage by analyzing
single-channel block kinetics between −125 and 50 mV.

Permeant ions knock off the blocker from its site
Block of open MthK decreased at negative membrane potentials due
to an acceleration of blocker off-rate (as seen by the reduced block
durations, Fig. 3a). At negative potentials, $K_{d^{\text{open}}}$ decreased exponen-
tially with voltage and showed similar slopes for all blockers,
suggesting a common mechanism (Fig. 3b, open symbols). Notably,
at positive potentials, blocker dissociation constants (or affinities)
were voltage independent.

Voltage-dependent channel block has often been modeled as the
charged blocker traversing a portion of the membrane electric field to
reach its binding site.44 The model predicts that voltage changes will
always modify block, unlike the plateau observed at positive voltages
(Fig. 3b). Furthermore, our kinetic analysis showed that blocker on-
rates were voltage independent; thus, the voltage dependence of blocker
affinity was exclusively from the off-rate (Supplementary Fig. 3).
This is consistent with a blocker “knock-off” effect, first described by
Armstrong,3 suggesting that blocker voltage dependence is not intrin-
sic but arises from coupling with permeant ions moving in the field.

Martinez-François and Lu formulated a model to describe how
blocker voltage dependence can arise from the blocker’s coupled move-
ment with permeant ions rather than from its intrinsic movement,36
a concept that has been occasionally explored.42,45–47 In this model
(Fig. 3c), the charged blocker does not bind within the membrane elec-
tric field but rather in close proximity (in the aqueous cavity) to the
permeant ions in the filter. At negative voltages, electrostatic repulsion

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between the blocker and permeant ions leads to permeant ion redistribution in the filter upon blocker binding, experimentally observed as voltage-dependent block (Fig. 3c, diagonal transition). At positive voltages, permeant ions are preferentially distributed farther from the blocker site, electrostatic repulsion is thus relieved and channel block is no longer voltage dependent (Fig. 3c, horizontal transition). This model predicts a blocker dissociation constant containing a voltage-dependent and a voltage-independent term ($K_d^{\text{open}}(V)$, equation (2)).

The permeant ion coupling model fits the voltage dependence of $K_d^{\text{open}}$ for all three blockers (Fig. 3b). At increasing negative voltages, the exponential term is dominant (equation (2)) and the blocker’s affinity decreases as the $K^+$ ions knock off the blocker from its site. At positive voltages, the exponential term is negligible and $K_d^{\text{open}}$ becomes essentially voltage independent. Our analysis of block during inactivation ($V \geq 50$ mV, Fig. 3b) is thus substantially simplified by the constraint that any voltage dependence in this range is due to gating.

**State-dependent block during MthK voltage gating**

In order to identify the location of the inactivation gate, we investigated how block was affected by voltage gating. We first measured a model-independent apparent dissociation constant, $K_d^{\text{app}}$ (equation (3)), for each blocker over the inactivation voltage range ($V \geq 50$ mV, Fig. 3b). Unexpectedly, the blockers exhibited opposite apparent affinity changes: inactivation decreased the apparent affinity for bTBA and bbTBA (increased $K_d^{\text{app}}$) but increased the apparent affinity for TBA (decreased $K_d^{\text{app}}$) (Fig. 3b).

Next, we examined which mechanisms of state-dependent block (Fig. 1) predict the observed apparent affinities (Fig. 3b). If the voltage gate is at the bundle crossing and the blocker can only bind to the open state (Scheme IIA, Fig. 4a), $K_d^{\text{app}}$ is predicted to increase with voltage according to $K_d^{\text{app}} = K_d^{\text{open}} P_c^{\text{control}}$ (Supplementary Note). This can explain the apparent affinity changes for bTBA and bbTBA but cannot describe the voltage-dependent

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**Figure 2** Block of open MthK. (a) Representative recorded currents through single MthK channels at −100 mV with TBA, bTBA and bbTBA (molecular models shown above traces). Closed level is indicated with a dashed line. (b) Closed and open dwell-time distributions for data in a. Control (top) was fit to a single closed-open model (right) to fit flicker gating (state F, Supplementary Fig. 1b). Blocker binding was fit with an additional closed state, OB (Scheme I). Fitted rates were used to calculate dissociation constants to MthK open state, $K_d^{\text{open}} = k_f k_{o}^{-1}$. The calculated $K_d^{\text{open}}$ values are: TBA, 570 µM; bTBA, 27 µM; and bbTBA, 3.8 µM. Black and red lines are exponential fits to distributions using the schemes (right).

**Figure 3** Voltage dependence of open-state block and apparent affinities during inactivation. (a) Single-channel traces without and with 25 µM bTBA at −25, −75 and −125 mV. (b) $K_d^{\text{open}}$ (open symbols) and $K_d^{\text{app}}$ (filled symbols; equation (3)) for TBA (black squares), bTBA (red circles) and bbTBA (blue triangles); n = 3–6, mean ± s.d., error bars smaller than symbols. $K_d^{\text{open}}$ data were fit to a model described in c (lines; equation (2)). TBA: $K_d^{\text{low}} = 0.37 \pm 0.03$ mM, $K_d^{\text{high}} = 0.16 \pm 0.01$ mM, $z = 0.87 \pm 0.01$; bTBA: $K_d^{\text{low}} = 2.0 \pm 0.5$ µM, $K_d^{\text{high}} = 1.7 \pm 0.2$ µM, $z = 0.67 \pm 0.03$; bbTBA: $K_d^{\text{low}} = 3.7 \pm 0.08$ µM, $K_d^{\text{high}} = 0.16 \pm 0.01$ µM, $z = 0.78 \pm 0.01$.

Dashed lines are extrapolated $K_d^{\text{open}}$ values. (c) Cartoon of $K^+$-blocker coupling model used to fit data in b (adapted from ref. 36). Left, pore with two conductive states differing in $K^+$ (green) distributions in selectivity filter (S1 and S3 or S2 and S4). Right, TBA (cyan) blocked MthK pore with $K^+$ in S1 and S3. Membrane voltage, $\Delta V_m$, is shown only across the selectivity filter. TBA binding with $K^+$ in S1 and S3 is voltage-independent with dissociation constant $K_d^{\text{low}}$ (horizontal transition), and TBA binding with $K^+$ in S2 and S4 is voltage-dependent with dissociation constant $K_d^{\text{open}} e^{-dV/T}$ (diagonal transition). Cartoon of ion movement between S1 and S3, and S2 and S4, is a simplification drawn merely for visualization of the ion-coupling concept.
Fit parameters are in Supplementary Tables 1–3 (see also Supplementary Fig. 4). (c) TBA state-independent access (Scheme III) fit results. Black squares are TBA $K_{\text{d}}^\text{open}$ measurements from Table 1 ($n = 3–6$, mean ± s.d.), and the black dotted line is the predicted $K_{\text{d}}^\text{open}$ using fitted parameters with Scheme III (equation (4), Online Methods). The green dotted line is the TBA $K_{\text{d}}^\text{open}$ fit from Figure 3 (equation (2)). The black solid and dashed lines are $K_{\text{d}}^\text{open}$ (63.5 ± 9.9 μM) and $K_{\text{d}}^\text{inact}$ (5.6 ± 0.6 μM), respectively, from fits to Scheme III ($n = 3$, mean ± s.d.).

Figure 4 Inactivation does not gate TBA access to the binding site. (a) Schemes used to fit single-channel kinetic data. (b) Example of closed dwell-time distributions from single MthK recordings with fits to the models in a: open-channel block (Scheme IIA, red dashed line), blocker trapping (Scheme IIB, green dotted line) and state-independent access models (Scheme III, black line). Fit parameters are in Supplementary Tables 1–3 (see also Supplementary Fig. 4). (c) TBA state-independent access (Scheme III) fit results. Black squares are TBA $K_{\text{d}}^\text{open}$ measurements from Figure 3 ($n = 3–6$, mean ± s.d.), and the black dotted line is the predicted $K_{\text{d}}^\text{open}$ using fitted parameters with Scheme III (equation (4), Online Methods). The green dotted line is the TBA $K_{\text{d}}^\text{open}$ fit from Figure 3 (equation (2)). The black solid and dashed lines are $K_{\text{d}}^\text{open}$ (63.5 ± 9.9 μM) and $K_{\text{d}}^\text{inact}$ (5.6 ± 0.6 μM), respectively, from fits to Scheme III ($n = 3$, mean ± s.d.).

The red and blue dashed lines are $K_{\text{d}}^\text{open}$ for TBA (2.9 ± 0.3 μM) and bbTBA (0.44 ± 0.02 μM), respectively, and gray dashed lines are $K_{\text{d}}^\text{inact}$ values for bTBA (7.5 ± 1.3 μM) and bbTBA (4.0 ± 0.9 μM) from fits to Scheme III ($n = 3$, mean ± s.d.).

Figure 5 bTBA and bbTBA block are consistent with state-independent access. (a) Examples of closed dwell-time distributions from single MthK recordings are shown for bTBA (top) and bbTBA (bottom) at 125 mV with fits to the open-channel block (Scheme IIA in Fig. 4a, red dashed line) and the state-independent access (Scheme III in Fig. 4a, black line) models.

Fit parameters are in Supplementary Tables 1–3 (see also Supplementary Fig. 5 and Supplementary Fig. 6). (b) bTBA (red) and bbTBA (blue) state-independent access (Scheme III in Fig. 4a) fit results. Red circles (bTBA) and blue triangles (bbTBA) are $K_{\text{d}}^\text{open}$ measurements from Figure 3 ($n = 3–6$, mean ± s.d.), and solid red and blue lines are calculated bTBA and bbTBA $K_{\text{d}}^\text{open}$ values using fitted parameters from Scheme III (equation (2), Online Methods). Red and blue dotted lines are bTBA and bbTBA $K_{\text{d}}^\text{open}$ fits from Figure 3 (equation (2)).

The red and blue dashed lines are $K_{\text{d}}^\text{open}$ for bTBA (2.9 ± 0.3 μM) and bbTBA (0.44 ± 0.02 μM), respectively, and gray dashed lines are $K_{\text{d}}^\text{inact}$ values for bTBA (7.5 ± 1.3 μM) and bbTBA (4.0 ± 0.9 μM) from fits to Scheme III ($n = 3$, mean ± s.d.).

TBA block locates the voltage gate at the selectivity filter

Using the trend in apparent affinity for TBA, we were able to narrow down the mechanism of block to either the gated-access model with trapping (Scheme IIB, Fig. 4a) or the state-independent access model (Scheme III, Fig. 4a). To distinguish between these two possibilities and the different gate locations they predict, we analyzed TBA microscopic block kinetics during gating. Single-channel kinetic data were acquired for a range of blocker concentrations and voltages for three independent bilayers (Fig. 4b and Supplementary Fig. 4). Data were globally fit to the three models (Schemes IIA, IIB and III, Fig. 4a). Scheme III, describing the state-independent access model, was the best fit to the data (solid black lines in Fig. 4b), clearly superior to the blocker-trapping model (green dotted lines) at high concentrations of TBA and high voltages as well as to the gated-access, open-channel block model (red dashed lines). Dwell-time distributions from all conditions are in Supplementary Figure 4, and fit parameters from all data sets are in Supplementary Tables 1–3. Confirming the visually better fits, Scheme III log-likelihood values were higher. These results show that closure of the voltage gate does not prevent TBA binding.

With our analysis, we were able to establish TBA affinity to the non-conducting, inactivated state, a conformation difficult to probe directly. Using the block parameters from Scheme III (Supplementary Table 3),...
we calculated the $K_d^{\text{open}}$, $K_d^{\text{inact}}$ and the predicted $K_d^{\text{dopen}}$ (Fig. 4c, equation (4), Online Methods). Unsurprisingly, the predicted $K_d^{\text{dopen}}$ was indistinguishable from the values determined experimentally. The $K_d^{\text{open}}$ of TBA from Scheme III also agreed with the value determined in the absence of inactivation (Fig. 4c, black line and green dotted line). The $K_d^{\text{inact}}$ of TBA (Fig. 4c, black dashed line) was an order of magnitude smaller than $K_d^{\text{open}}$. Thus, TBA not only accesses both the open and inactivated states but also binds to the inactivated state an order of magnitude more strongly than to the open state, unambiguously indicating that the voltage gate is above the blocker binding site, at the selectivity filter.

bTBA and bbTBA block support state-independent access

Can bTBA and bbTBA also bind to the inactivated MthK pore? Figure 3b showed that unlike TBA, the apparent affinities for bTBA and bbTBA decreased as the MthK channel inactivated, suggesting perhaps a different mechanism. To investigate, we analyzed single-channel data sets acquired over a range of intracellular blocker concentrations and voltages for bTBA and bbTBA. We found that the state-independent access model (Scheme III, Fig. 4a) fits the data in all conditions, whereas the open-channel block model (Scheme IIa, Fig. 4a) failed to fit the data at high levels of inactivation (Fig. 5a).

Dwell-time distributions from all experimental conditions are in Supplementary Figure 5 and Supplementary Figure 6, and fit parameters from three data sets each for bTBA and bbTBA for Schemes IIa and III in Figure 4a are in Supplementary Tables 1–3.

Using the block parameters from Scheme III (Supplementary Table 3), we calculated the $K_d^{\text{open}}$, $K_d^{\text{inact}}$ and the predicted $K_d^{\text{dopen}}$ for bTBA and bbTBA (Fig. 5b). $K_d^{\text{dopen}}$ and $K_d^{\text{open}}$ values agreed with values determined experimentally. $K_d^{\text{inact}}$ was an order of magnitude larger than $K_d^{\text{open}}$ for bbTBA but only twice as large for bTBA. Thus, the decrease in apparent affinities for bTBA and bbTBA during inactivation is not due to gated access but results from lower affinities to the inactivated state.

We also attempted to fit these data to the blocker-trapping model (Scheme IIb, Fig. 4a), but the parameters describing trapping (between OB and IB) were not well constrained and gave inconsistent results from channel to channel (not shown). We concluded that these data were not optimal for evaluating this model, likely reflecting our conclusion that blocker affinity for the inactivated state was less than that for the open state. We also reasoned that if TBA does not get trapped inside the channel (as we showed above), then the larger blockers, bTBA and bbTBA, would likely not be trapped either. Consequently, the high-quality fits to the state-independent access model argue that all three blockers can bind to the inactivated state, strongly supporting voltage-dependent gating at the selectivity filter.

Inactivation alters the binding kinetics of each blocker

We have shown that three quaternary ammonium blockers bind differently to the open and inactivated states. We have also analyzed an additional blocker, tetraptethylammonium (TPEa), over a limited set of conditions (Online Methods, Supplementary Fig. 7 and Supplementary Table 3) to supplement our analysis of blocker state dependence. The differences in microscopic block kinetics between open and inactivated states suggest underlying conformational changes in the MthK pore during voltage gating.

Inactivation decreased the on-rate constant for all four blockers by 50–75% (Fig. 6a). This suggests the barrier that the blocker has to cross to enter the channel pore is greater for the inactivated state than for the open state. On the other hand, inactivation modified the off-rate in a blocker-dependent manner (Fig. 6b). For TBA, the off-rate decreased by more than an order of magnitude during inactivation, overwhelming the decrease in on-rate and accounting for the large increase in affinity (decrease in $K_d$) relative to the open state (Fig. 6c). For TPEa, the off-rate was reduced by ~80%, resulting in a small increase in affinity upon inactivation. For bTBA, the off-rate was essentially state independent, and the affinity change during inactivation was entirely a result of the decrease in on-rate. For bbTBA, inactivation increased the off-rate by ~250%, decreasing the affinity (increasing the $K_d$) relative to the open state. Therefore, both tetraptethylammonium blockers, TBA and TPEa, bound more strongly to the inactivated state, whereas the blockers with attached benzyl rings, bTBA and bbTBA, bound more strongly to the open state.

Table 1 Data collection and refinement statistics (molecular replacement)

|                      | MthK | MthK-TBSb |
|----------------------|------|-----------|
| **Data collection**  |      |           |
| Space group          | P1   | P1        |
| Cell dimensions      |      |           |
| a, b, c (Å)          | 44.03, 63.45, 63.48 | 43.97, 64.30, 64.38 |
| α, β, γ (°)          | 90.03, 89.99, 89.99 | 89.99, 89.95, 90.00 |
| Resolution (Å)       | 44.9–1.65 | 44.0–1.70 |
| α (Å)                | (1.71–1.65) | (1.76–1.70) |
| Rfree                | 4.2 (39.7) | 4.8 (67.7) |
| completeness (%)      | 96.3 (93.8) | 96.4 (93.2) |
| redundancy            | 3.2 (3.0) | 3.3 (3.1) |
| **Refinement**       |      |           |
| Resolution (Å)       | 44.86–1.65 | 36.14–1.69 |
| No. reflections      | 78,558 | 73,246 |
| Rwork / Rfree        | 0.164 / 0.181 | 0.173 / 0.191 |
| No. atoms            |      |           |
| Protein              | 5,240 | 5,224     |
| Ligand/on            | 104  | 168       |
| Water                | 266  | 216       |
| B factors            |      |           |
| Protein              | 21.8 | 29.8      |
| Ligand/on            | 47.6 | 58.2      |
| Water                | 33.3 | 39.4      |
| r.m.s. deviations    |      |           |
| Bond lengths (Å)     | 0.01 | 0.009     |
| Bond angles (°)      | 1.173 | 1.112     |

Values in parentheses are for highest-resolution shell.
Figure 7 TBSb difference density in the cavity supports blocker binding immediately below the selectivity filter. Diffraction data for crystals of the isolated MthK pore with and without the heavy TBSb blocker were used to generate an $F_{o,TBSb} - F_{o,no blocker}$ difference map (red mesh), shown in the channel cavity, contoured at 4.5 $\sigma$. This peak precisely overlapped with a previously modeled TBA molecule (shown right) found in the cavity of KcsA, positioned by aligning the selectivity filter structures of the TBA-KcsA complex (2HVK) with MthK. Two opposing subunits of the MthK channel pore structure are shown in gray with four potassium ions in the selectivity filter (green spheres).

Blockers bind immediately below the selectivity filter
The results above strongly indicate that the voltage gate is located above the binding site for quaternary ammonium blockers in the MthK pore. The binding sites within other K$^+$ channels are known to be in the aqueous cavity$^{4,8}$. However, in order to unequivocally locate the voltage gate, we attempted to directly observe bound blocker inside the open MthK pore using X-ray crystallography$^{34}$. High blocker concentrations in the crystals (~10 mM) did not result in interpretable blocker density when compared to the blocker-free pore electron density maps. We concluded that either the blocker binding is highly disordered or the occupancy of bound blocker is very low inside the crystal. This last possibility may arise from the detergent, precipitant or other crystal properties that may antagonize blocker binding.

In order to maximize the likelihood of observing bound blocker even at low occupancy, we collected diffraction data from MthK pore crystals containing the heavy tetrabutyl antimony, TBSb. We constructed multiple difference maps using several data sets from MthK-TBSb crystals and MthK-no-blocker crystals. Figure 7 shows an example $F_{o,TBSb} - F_{o,no blocker}$ difference map contoured at 4.5 $\sigma$. Data collection and model refinement statistics for the crystals used to make the map in Figure 7 are given in Table 1. A single difference peak was observed in the maps at a site below the selectivity filter that aligned perfectly with a previously modeled TBA binding site in KcsA$^{48}$. The strength of this difference peak was consistent with very low blocker occupancy within the crystal. However, the agreement with previous crystallographic evidence using the closed KcsA channel strongly suggests a conserved quaternary ammonium binding site immediately below the selectivity filter.

DISCUSSION
In the absence of activating Ca$^{2+}$, MthK channels were proposed to close at an intracellular-facing gate analogous to the bundle crossing in the KcsA closed structure$^{10}$. It was conceivable that voltage-dependent gating of MthK also occurred at this location. A previous report on MthK voltage-dependent gating, though, had demonstrated gating sensitivity to external K$^+$ concentration and had suggested that the voltage dependence arises from ion occupancy of the selectivity filter$^{35}$, similar to C-type inactivation in K$_v$ channels. However, a coupling mechanism between selectivity filter and an intracellular-facing gate was also a possibility.

We used quaternary ammonium blockers as state-dependent accessibility probes to investigate the physical location of the voltage-dependent gate within the pore of MthK channels. Unlike related studies of K$_v$$^{5,6}$, BK$^{26–28}$, HCN$^{48}$ and CNG$^{21,49}$ channels, we exclusively used kinetic analysis of single-channel block to demonstrate that the voltage gate is at the selectivity filter.

Blocking studies of voltage-gated ion channels require careful consideration of voltage-dependent contributions from the movement of charged blockers and the gating of the channel$^{31,26}$. Numerous studies of BK channels—eukaryotic homologs of MthK channels that have been proposed to gate at the selectivity filter—highlight these challenges. Aldrich and co-workers showed that BK channel block by bbTBA displayed a voltage dependence that appeared to be consistent with gated access but was shown to result from the charged blocker itself, and they eventually concluded that bbTBA had state-independent accessibility to the BK pore$^{28}$. This interpretation was later questioned by a modeling study that agreed with the binding of bbTBA to closed BK channels but suggested that partial activation of BK was required for bbTBA binding$^{28}$. These studies highlight the difficulty in dissecting voltage-dependent gates, state-dependent affinities (due to conformational changes), voltage-dependent affinities (due to permeant ion coupling) and possible coupling between two gates. In the case of MthK, we were able to separate voltage-dependent changes in blocker affinity due to permeant ions (at negative voltages) and voltage-dependent changes due to inactivation gating (at voltages above 50 mV) by showing that they occur over different voltage ranges. This was a major advantage in our modeling and interpretation of the data as we were able to assume that the blocker affinities to the open and inactivated states were voltage independent during inactivation (50–125 mV).

For the MthK open-channel block data in this study, we characterized blocker on- and off-rates as a function of voltage and found that voltage-dependent block was almost entirely due to blocker knock off. That is, the on-rates were essentially voltage independent, likely reflecting the absence of a substantial voltage drop across the pore cavity below the selectivity filter. This is an important finding because it provides experimental evidence that the fractional voltage drop across the cavity is very small for MthK, consistent with evidence for CNG channels and calculations using continuum electrostatics$^{50,51}$. Other channels with similarly large or larger intracellular cavities, such as BK$^{29,52}$, also likely focus the membrane electric field entirely across the selectivity filter.

We determined that blockers access the MthK cavity in both open and inactivated states. The analysis of several blocker molecules was crucial, as only TBA proved ideal in distinguishing between the state-independent access and the blocker-trapping models, which led to a definitive location of the gate. Unlike TBA, both TPeA and bbTBA showed a reduced affinity upon channel inactivation, which compromised their utility in directly ruling out blocker trapping. We reasoned that these larger blockers were less likely to be trapped than the smaller TBA, which we showed was not trapped. The block kinetics for all blockers tested were very well accounted for by state-independent access to the binding site.

Does inactivation involve changes in the K$^+$ channel beyond the narrow confines of the selectivity filter? From our kinetic analysis (Figs. 4–6), we obtained blocker on- and off-rates to both open and inactivated MthK for four blockers (TBA, TPeA, bbTBA and bbTBA). To our knowledge, only one blocker affinity has been reported for an inactivated K$^+$ channel conformation$^{29}$. The kinetic basis for state-dependent block ($K_{d,open} \neq K_{d,inact}$), summarized in Figure 6, suggests structural changes in MthK beyond the selectivity filter during voltage gating. The on-rates for all four blockers were lower in the inactivated than in the open state, suggesting that they have reduced access to
the inactivated pore. Perhaps a small narrowing at the entrance to the pore accompanies inactivation.

The state dependence of blocker off-rate was blocker specific. The inactivated pore binds TBA and TPEA for longer (off-rates are slower in the inactivated state) than the open pore, whereas the open pore binds bTBA for longer (off-rates are faster in the inactivated state). bTBA was the only blocker for which the off-rate was state independent. These changes contribute to the final state-dependent affinities (Fig. 6c). In the absence of structural data, it is difficult to speculate why different blockers vary in their state-dependent off-rates. It is possible that a conformational change such as a rotation or tilt of inner helices occurs in the pore below the selectivity filter during inactivation. Perhaps this conformational change enhances or disrupts the blocker-specific hydrophobic contacts with the protein. Other studies have indicated that selectivity filter gating may induce changes in the channel cavity or intracellular entryway20,27,29,53–55, and in K+, channels, for example, this may be related to coupling mechanisms between two gates19,56. In the context of ligand-gated channels such as MthK, we speculate that changes between open and inactivated conformations may reflect the allosteric connection between cytoplasmic ligand-binding domains and a selectivity filter gate.

The location of the quaternary ammonium binding site within the pore of K+ channels was previously modeled for the closed KcsA channel using X-ray crystallography37–39. Recently, a high-resolution structure of the isolated open MthK pore was solved44. Unfortunately, our attempts to recrystallize the MthK pore with blockers did not result in convincing electron density for modeling blocker binding. The effort was salvaged using a heavy-atom TBA analog, TBSb4, that established the binding location immediately below the selectivity filter with an electron density difference peak from a Fo − Fc blocker map. Importantly, our fundamental conclusion that the gate is within the selectivity filter does not depend on a precise molecular model of blocker binding.

In summary, we explored voltage-dependent block of the model MthK K+ channel by four quaternary ammonium ions. The voltage dependence of open-channel block was consistent with a model of blocker coupling to permeant K+ ions. TBA was an ideal probe for analyzing state-dependent block during voltage gating as the intrinsic voltage dependence of the block occurred over a different voltage range than the voltage-induced changes in the inactivation gate of MthK. Kinetic analysis revealed that blockers could access both open and inactivated states, although with different affinities. These results suggested possible structural changes in the pore below the selectivity filter but ruled out gated access to the block site. Crystallographic evidence using TBSb supported the picture of blocker binding below the selectivity filter, further establishing the location of the voltage-dependent inactivation gate at the selectivity filter.

METHODS

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

Accession codes. Protein Data Bank: Models and structure factors for the MthK (S68H V77C) pore crystallized in the absence and presence of TBSb have been deposited under accession codes 4HYO and 4HZ3, respectively.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

D.J.P. and C.M.N. designed the research; D.J.P. acquired and analyzed single-channel data; D.J.P. and J.G.M. acquired and analyzed crystallographic data; D.J.P., J.G.M. and C.M.N. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

MthK purification and reconstitution. MthK channels were expressed, purified and reconstituted into proteoliposomes using protocols described previously\(^{33}\). All reagents were from Sigma unless otherwise indicated. Channels were purified in protein buffer (100 mM KCl, 20 mM HEPES, pH 7.6), with 5 mM N-decyl-\(\beta\)-maltopyranoside (DM, Affymetrix) and reconstituted into liposomes of 3:1 POPE:POPG (Avanti Polar Lipids). Lipids were solubilized in reconstitution buffer (400 mM KCl, 5 mM N-methyl-D-glucamine, 10 mM HEPES, pH 7.6) with 34 mM CHAPS (Affymetrix). Liposomes were formed and detergents were removed by passing MthK and lipids (1–5 µg protein per mg lipid) over a 20 ml Sephadex G50-fine column (GE Healthcare) using reconstitution buffer. Eluted liposomes were flash frozen in liquid nitrogen and stored at \(-80^\circ\)C.

MthK pore-only crystallization. The pore of MthK (wild-type or S68H V77C) was isolated from the intracellular domains and crystallized as previously described\(^{34}\). Trypsin type I (Sigma, T8003) was added to purified MthK at 1:50 trypsin:MthK (wt:wt) for 2 h. Trypsin inhibitor type II-\(\alpha\) (Sigma, T9253) was added at 2:1 inhibitor:trypsin (wt:wt), and digested protein was purified on a Superdex 200 column using protein buffer with 5 mM DM. The 14.3 ml peak was purified with a second Superdex 200 column using crystallization buffer (100 mM KCl, 10 mM MOPS, pH 8.0) with 10 mM Anagrade LDAO (Affymetrix). Protein was concentrated to 15 mg ml\(^{-1}\) (assuming 1 O.D.\(_{280}\) = 1 mg ml\(^{-1}\)). Crystals formed at 20 °C in sitting drops of 1.5 µl protein mixed with 1.5 µl well. Well solution was 3.5–4.0 M 1,6-hexanediol (Hampton Research), 100 mM MES, pH 6.5. For tetrabutyl antimony (TBSb) cocrystallization, 5–10 mM TBSb and account for the asymmetric TBSb molecule (data can also be processed in resolution ranged from 1.65 to 2.0 Å. Data were processed using HKL2000 (HKL of difference density with a maximum peak at 5–8\(\sigma\).

Crystal diffraction data collection and analysis. MthK pore crystals were cryo-preserved in liquid N\(_2\), and diffraction data from 6 crystals with and without TBSb were collected at 93 K using a wavelength of 1.1 Å at the X25 beamline at the National Synchrotron Light Source, Brookhaven National Laboratory. Their resolution ranged from 1.65 to 2.0 Å. Data were processed using HKL2000 (HKL Research) in space group P1 to reduce noise along the fourfold channel axis and account for the asymmetric TBSb molecule (data can also be processed in P4\(_2\)2\(_1\), as previously reported\(^{34}\)). Molecular-replacement phases for these data were determined with Phenix\(^{35}\) using pdb 3LDC\(^{34}\), and models were generated through rounds of refinement in Phenix and model building in CoVal\(^{36}\). All residues fit into the favored region of the Ramachandran plot. Data collection and refinement statistics for two deposited models are given in Table 1. Ordered solvent molecules and ions were previously modeled into densities observed in the MthK pore cavity without blockers (pdb 3LDC). The presence of TBSb (\(\approx 50\) µM) did not result in the large expected increase in electron density, potentially due to low occupancy. Consequently, we calculated a difference map between the TBSb-containing and TBSb-free data (\(F_{\text{TBSb}} - F_{\text{no blocker}}\)) using phases calculated from the TBSb-free structure model (Table 1) with programs from the CCP4 suite\(^{39}\). Eleven other difference maps (not shown) also revealed a region of difference density with a maximum peak at 5–8 \(\sigma\) located below the selectivity filter. Structural alignment with a model of KcsA with TBA, pdb 2HVK\(^{38}\), shows that the modeled TBA site overlaps with our calculated difference density. The map in Figure 7 was contoured at 4.5 \(\sigma\) in PyMOL. Structural alignment with the published MthK model used for molecular replacement, pdb 3LDC\(^{34}\), shows that the TBSb antimony is located 2.1 Å below an ordered water, modeled below the selectivity filter.

Single-channel recording in lipid bilayers. MthK proteoliposomes were applied to horizontal lipid-decane bilayers (3:1 POPE:POPG in decane) painted across the selectivity filter. The selectivity filter.

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on-rate constants to the open and inactivated states, respectively. Blocker off-rates are $k_{\text{off}}$ and $k_{\text{offi}}$. State-dependent dissociation constants are $K_{d}^{\text{open}} = k_{\text{off}} k_{\text{on}}^{-1}$ and $K_{d}^{\text{inact}} = k_{\text{offi}} k_{\text{on}}^{-1}$. Three independent single channels for TBA (Schemes IIA, IIB and III), bTBA and bbTBA (Schemes IIA and III only), were analyzed. For tetrabutylammonium (TPeA), only one blocker concentration was recorded for 50–125 mV; the data were globally fit to Scheme III only and the process was repeated for three independent channels. The voltage-dependent inactivation was initially constrained in the global fits to values found for no-blocker experiments (Supplementary Fig. 1a). These constraints were then released for finding the best fit. The measured $K_{d}^{\text{rep}}$ values from 50 to 125 mV (equation (2)) were compared with the calculated $K_{d}^{\text{rep}}$ values for Scheme III using mean values for the fit parameters and the following expression (equation (4)); see Supplementary Note for derivation):

$$K_{d}^{\text{rep}}(V) = \frac{\frac{V}{R} e}{1 + \frac{V}{R} e} K_{d}^{\text{open}} + K_{d}^{\text{inact}} \frac{(V/R)^2}{k_2 e} K_{d}^{\text{inact}}$$

We also fit the data to a cyclic version of Scheme III that includes voltage-dependent connections between states OB and IB. These additional transitions represent inactivation gating in the presence of bound blocker. However, these rates were not well constrained and were typically fit to values approaching zero (not shown). Because the final conclusion about the gate location is unaltered by this detail, we used Scheme III to describe state-independent blocker access.

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