Integrating spatiotemporal features of a ligand-regulated, multi-state allosteric protein

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Dynamic protein molecules are defined by their spatiotemporal characteristics and should thus be represented by models incorporating both characteristics. Structural biology enables determination of atomic structures of individual conformational states of a given protein. Obtaining the complementary temporal information of a given time resolution, which can be directly linked to the corresponding atomic structures, requires identifying at each time point the specific conformational state adopted by the protein. Here, we examine individual regulator of conductance to K+ (RCK) domains in the regulatory module of the MthK channel by monitoring in real time the orientation of an α-helix that is conformational-state-specific. The acquired dynamic information that specifies an RCK domain’s multi-state conformational changes, combined with already available corresponding atomic structures, enables us to establish an experiment-based spatiotemporal representation of an RCK domain, and to deduce a quantitative mechanistic model of the channel.

Most proteins undergo functionally important conformational changes. These changes are defined in terms of their spatial and temporal characteristics, the knowledge of which is required to achieve a full mechanistic understanding of a protein. Structural biologists have determined ångström-resolution three-dimensional structures of individual conformational states for a large number of proteins. Recent advances in cryo-electron microscopy are accelerating the process of structure determination. To complement this progress in structural biology and to transition from a static to a dynamic approach, we need relatively general approaches to directly determine the lifetimes of individual conformational states of a given protein and the connectivity among the states over the observed time. These temporal measurements can be used to relate individual structural states, only if the measurement at each time point also contains the spatial information that can unambiguously identify the specific conformational state adopted by the protein.

We currently lack general, effective techniques to directly examine protein-conformational kinetics at a single-molecule level, in a manner that allows the unambiguous linking of a given kinetic state at each time point to a specific structural conformation among multiple possible conformations that the protein may adopt. This deficiency is partly because protein-conformational changes are not only rapid but also often occur on an ångström scale, which is too small to be reliably and quantitatively resolved in real time. Protein-conformational changes have been probed using a fluorescence resonance energy transfer (FRET)-based method, which allows the deduction of distance between a fluorescence donor and an acceptor, generally on a nanometer scale. However, such a deduction requires knowledge of the actual fluorescence transfer efficiency, which is often difficult to assess because it depends on the local environment and, in most cases, on the relative orientation of the fluorophore pair. When a protein exists in multiple states, resolution and unambiguous identification of conformational states are even more challenging.

By quantitatively resolving fluorescence-polarization changes of a bifunctional fluorophore attached to a protein, we are able to monitor the protein’s multi-state ångström-scale conformational changes on a millisecond scale. Our test protein is the regulatory module of the MthK channel, formed by two stacked ring-like structures, each of which comprises four RCK domains. RCK can adopt three conformational states, S1, S2, and S3, which have been determined crystallographically. Ca2+ binds to RCK domains and regulates the conformational changes in the regulatory module, activating the channel by markedly shortening the lifetime of the closed state and modestly lengthening the lifetime of its open state. Single-channel current-recording approaches allow us to follow the open or closed states of the channel gate but not the conformational state of its regulatory module. Our fluorescence-based study has revealed that, in the isolated regulatory module, individual RCK domains independently transition among S1, S2, and S3, and Ca2+ binding to RCK promotes S2 over S1 and S3 (ref. 6; all notation is listed in Supplementary Note 1). A minimal model of six states, three with and three without Ca2+ bound ions, is required to account for the behavior of RCK. On the basis of the characteristics of RCK, we deduced an energetic model that quantitatively predicts the dependence of the channel’s open probability (p) on the Ca2+ concentration.

Here we set out to determine the temporal characteristics of RCK’s conformational states and integrate them with the existing structural information, thereby creating a spatiotemporal representation for RCK, from which we deduce a mechanistic model for the whole channel.

Results

Dynamic behaviors of an RCK domain. To examine conformational dynamics of the RCK domain, we monitored helix αB that is located closest to the channel gate and adopts a unique spatial orientation in each of the three states determined crystallographically. We monitored the polarized fluorescence emitted by a single bifunctional rhodamine molecule, attached to the α-helix in one of the eight RCK domains within the isolated regulatory module. Figure 1b shows consecutive images of the emission from a single fluorophore attached to a regulatory module, recorded under a (nominal) Ca2+-free condition with a camera over a constant 20 ms interval per frame, which was split according to 0°, 45°, 90°...
and 135° polarizations. The integrated values of the four intensities (Fig. 1c) were used to calculate inclination (θ) and rotation (φ) angles of the fluorophore’s dipole in a particular conformational state, as well as the overall angle change (Ω) between two conformational states (Fig. 1d). The lines in Fig. 1c, d, color-coded for states, were generated by setting the amplitude of a given event uniformly to the average of the observed values within that event, a procedure that enhanced the effective signal-to-noise ratio and thus angle resolution on the basis of events. Starting and end points of a particular state (that is, an event) were determined from concurrent changes among four intensities (Fig. 1c), with a method based on the changepoint algorithm, and the specific state, S1, S2 or S3, was identified using a method based on a k-mean-clustering algorithm (Methods; ref. 4).

From Ω traces, containing both θ and φ information, we obtained temporal information regarding RCK’s conformational changes where the three tracked RCK conformations are represented by the corresponding electron density maps (PDB 1LNQ and 2FY8)6,7, in accordance with the temporal information of the Ω trace (d). The tracked RCK is presented against a contour of the regulatory module in a side view of the regulatory domain (top) or in a view along its central axis (bottom).

**Ca2+-dependent conformational kinetics of RCK.** An experiment-based kinetic model that satisfactorily accounts for the observed conformational changes of RCK is the temporal basis for building a spatiotemporal model, defined by the number and connectivity of states, the rate constants for all state transitions and the K_D values of Ca2+ for individual states. We describe below how we determine those rate constants from the observed state dwell times, based on the probabilities of individual state-to-state transitions. For clarity, we use the terms of dwell times and lifetime to refer to individual measurements and their mean value, respectively.

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**Fig. 1** Structures of RCK, polarized-fluorescence intensities, dipole orientations of a fluorophore, and video frames for the tracked RCK. a, Three RCK conformations are aligned and the α-helix under examination is color-coded as indicated below; the color scheme for S1, S2 and S3 states is used throughout. The reference coordinates are shown below the structures. b, Seventeen consecutive frames of four intensity components (I_0, I_45, I_90 and I_135) recorded from a fluorophore attached to a single RCK domain at a constant interval of 20 ms per frame during a selected 0.34-s segment of a ~4-s recording. c, Intensity values, in arbitrary units (AU), were integrated from the 17 images shown in b and plotted against time. d, Values of θ and φ calculated from the four intensity traces (c) and values of Ω calculated relative to S2 in c and d, transition points of the colored traces superimposed on the records were determined by a changepoint analysis, and the constant values between transition points are arithmetic means of the observed data. e, Consecutive frames of Supplementary Video 1 of RCK conformational changes where the three tracked RCK conformations are represented by the corresponding electron density maps (PDB 1LNQ and 2FY8)6,7, in accordance with the temporal information of the Ω trace (d). The tracked RCK is presented against a contour of the regulatory module in a side view of the regulatory domain (top) or in a view along its central axis (bottom).
Fig. 2 | Observed dwell-time distributions and lifetimes of RCK conformations, and a kinetic model of RCK. a. Dwell-time distributions of the three conformational states, obtained in the presence of four indicated Ca\textsuperscript{2+} concentrations. The curves superimposed on the data were calculated from lifetimes using a single-exponential function as described in the text. b. Lifetimes (mean ± s.e.m., 20–1,003 events per distribution) of individual states in the presence of various Ca\textsuperscript{2+} concentrations, calculated from the observed dwell times. The curves superimposed on the data correspond to equation (17) (Supplementary Note 3). c. State diagram of a kinetic model of RCK, based on an energetic study\textsuperscript{5}, where \( K_n \) and rate constants of individual state transitions are denoted.

Figure 2a shows the distributions of individual dwell times of RCK observed in \( S_i \), \( S_j \), or \( S_k \) for four \( \text{Ca}^{2+} \) concentrations, measured from data traces similar to those shown in Fig. 1. If the dwell times follow a single-exponential distribution, the maximum-likelihood estimate of the lifetime (\( \bar{\tau}_i \)) of state \( i \) can be calculated using the arithmetic mean of individual dwell times. We used an equation for data recorded with a camera at a constant frame rate (equation (13) in Supplementary Note 2), which also addresses the problem arising from missing short events\textsuperscript{12}. We directly calculated the \( \bar{\tau}_i \) value for each state in a given \( \text{Ca}^{2+} \) concentration and, for illustration, we also calculated single-exponential curves (Fig. 2a) from \( \bar{\tau}_i \) using equation (12) (Supplementary Note 2). All resulting \( \bar{\tau}_i \) values are plotted against the \( \text{Ca}^{2+} \) concentration in Fig. 2b.

In the absence of \( \text{Ca}^{2+} \), the lifetime of a given state is defined by the rate constants of transitions from that state to its two neighboring states (Fig. 2c, left). We can determine the rate constants for those transitions from their relative probabilities and lifetimes. For example, \( S_i \) might transition to either \( S_j \) or \( S_k \) with probability \( p_{i,j} \), or \( p_{i,k} \). For these parallel transitions, the total effective rate of exiting \( S_i \) \( (k_j) \) would be the sum of rate constants \( k_{i,j} \) and \( k_{i,k} \). Generally, the rate \( (k_i) \) of state \( S_i \) transitioning to either of its neighboring states \( S_j \) or \( S_k \) together is expressed as

\[
k_i = k_{i,j} + k_{i,k} = \frac{1}{\bar{\tau}_i} \quad (1)
\]

where \( k_{i,j} \) and \( k_{i,k} \) manifest themselves in accordance with

\[
k_{i,j} = \frac{1}{\bar{\tau}_j} p_{i,j} \quad \text{and} \quad k_{i,k} = \frac{1}{\bar{\tau}_k} p_{i,k} \quad (2)
\]

Rate constants \( k_{i,j} \) and \( k_{i,k} \) could therefore be determined from the observed lifetime \( \bar{\tau}_i \) in a \( \text{Ca}^{2+} \)-free condition. Once the respective state-to-state transition probabilities \( (p_{i,j}) \) were determined. We calculated \( p_{i,j} \) (Fig. 3a) from the ratio of the number of \( S_i \)-to-\( S_j \) transitions \( (n_{i,j}) \) and the total number of \( S_i \) events \( (N_i = n_{i,j} + n_{i,k}) \).

\[
p_{i,j} = \frac{n_{i,j}}{N_i} \quad \text{and} \quad p_{i,k} = \frac{n_{i,k}}{N_i} \quad (3)
\]

From the above relations, we obtained the following equation for direct calculation of \( k_{i,j} \) from experimentally determined \( \bar{\tau}_j \), \( n_{i,j} \) and \( N_i \):

\[
k_{i,j} = \frac{1}{\bar{\tau}_j} \frac{n_{i,j}}{N_i} \quad \text{and} \quad k_{i,k} = \frac{1}{\bar{\tau}_k} \frac{n_{i,k}}{N_i} \quad (4)
\]

On the basis of these relations, we calculated rate constants for all \( \text{Ca}^{2+} \) concentrations (Fig. 3b). We could obtain the rate constants \( k_{i,j} \) and \( k_{i,k} \) for the \( \text{Ca}^{2+} \)-free and \( \text{Ca}^{2+} \)-bound forms \( (S_i \text{ and } S_{j,k}\text{Ca}^{2+}) \) either from apparent lifetimes \( \bar{\tau}_i (1/p_{i,j,k}) \) for zero and saturating \( \text{Ca}^{2+} \) conditions or from analyzing the entire plots in Fig. 3b. Because the \( \text{Ca}^{2+} \)-binding process is generally near the diffusion limit, \( S_i \) and \( S_{i,k}\text{Ca}^{2+} \) should be in rapid equilibrium such that the \( \text{Ca}^{2+} \)-binding kinetics would be irrelevant within the time scale of the present measurements. Thus, when \( S_i \) transitions to \( S_j \), observed \( S_i \) \( (\text{obs}S_i) \) might have been in either
a Ca$^{2+}$-free (S) or Ca$^{2+}$-bound (S,Ca$^{2+}$) state. In the presence of Ca$^{2+}$, the apparent rate constant ($k_{0,i}$) for state S transitioning to $S_i$ can be expressed as

$$k_{0,i} = \frac{k_{ij} + Ca^{2+}K_{Di}}{1 + Ca^{2+}K_{Di}}$$

(5)

where the $K_{Di}$ of a given state $i$ is defined by

$$K_{Di} = \frac{[S_i]Ca^{2+}K_{ni}}{[S_i](Ca^{2+})_{ni}}$$

(6)

Fitting equation 5 to all plots in Fig. 3b yielded the 12 rate constants in the model shown in Fig. 2c, with or without Ca$^{2+}$ bound ions ($k_{ij}$ and Ca$^{2+}k_{ij}$; Supplementary Table 1). To maintain a common set of $K_{Di}$ values between kinetic and thermodynamic analyses, we constrained them with those obtained from analyzing the Ca$^{2+}$ dependence of state probabilities (Supplementary Table 2).

**A spatiotemporal model of the isolated regulatory module.** With the temporal information and existing structural data of RCK's conformational states in hand, we can now create an integrated spatiotemporal model for the eight RCK domains that independently undergo conformational changes in an isolated regulatory module. To produce a set of temporal templates, we simulated the time courses of concurrent, independent transitions for eight RCK domains in different Ca$^{2+}$ concentrations. For a given Ca$^{2+}$ concentration, the initial state in the simulation was randomly drawn from a trinomial distribution of the three states, $S_1$, $S_2$ and $S_3$, which was built according to probabilities $p_{11}$, $p_{12}$ and $p_{13}$ (calculated as previously described). From that initial state (say $S_i$), a simulated dwell time (t$_{sim}$) was obtained by randomly drawing from an exponential dwell-time distribution defined by $-1/k_{Sim}$ (calculated with equation (17) in Supplementary Note 3, with the parameters as specified in Supplementary Table 1). As the simulation progressed in constant discrete 20-ms increments, the RCK domain remained in $S_i$ until the accumulated time of this event was greater than or equal to t$_{sim}$. At this point, the RCK domain in $S_i$ transitioned to either $S_j$ or $S_k$, based on the outcome of a random draw from a binomial distribution defined by the relative probabilities of $S_j$ exiting to $S_i$ versus $S_k$ ($p_{i,j}$ or $p_{i,k}$; equation 3). The t$_{sim}$ of this second event and its termination point were determined as described above. These steps were repeated until the entire simulation was completed. At a given Ca$^{2+}$ concentration, Ca$^{2+}$ occupancy in an RCK domain was determined by a random draw from a binomial distribution defined by the probabilities ($p_{bi}$ and 1 – $p_{bi}$) of the Ca$^{2+}$ site being occupied and not occupied:

$$p_{bi} = \frac{1}{1 + Ca^{2+}K_{ni}}$$

(7)

Figure 4a shows a 5-s segment of a 30-min simulated time course of state-to-state transitions for each of the eight RCK domains (A–H; Fig. 4b) in the regulatory domain in 0 mM Ca$^{2+}$. Repeating this for all experimental Ca$^{2+}$ conditions, we calculated the $\tau_{si}$ for each of the six transitions of RCK from the 100,000 simulated data points (Fig. 4c). For all Ca$^{2+}$ concentrations examined, the calculated lifetimes from the simulations (open symbols) reasonably matched the corresponding experimental data points (closed symbols). The state probabilities calculated from the simulations were also comparable to experimentally determined ones (Fig. 4d). This general good agreement between experimental data and outcomes of simulations validates the simulated temporal templates.

**Fig. 4 | Simulated kinetics and video frames for individual RCKs in the isolated regulatory module.** a, b, Concurrent simulations of state transition kinetics of eight independent RCKs for a Ca$^{2+}$-free condition (a, A–H) as assigned in the structure model (b), created according to the three conformations documented in PDB 1LNQ and 2FY8, and shown in a side view (upper) and a view along its central axis (lower); individual states are color-coded as indicated. c, d, Time constants for individual state-to-state transitions and the probabilities of RCK’s three conformational states calculated from simulated data (open symbols), compared to experimentally observed data points (closed symbols; mean ± s.e.m, 20–1,003 events per distribution). e, Consecutive frames of a video segment of the regulatory module in the two views (Supplementary Video 2), generated as described in the text.

Together, the kinetic information obtained here and crystal structures already available together allowed us to create an experiment-based, virtual spatiotemporal ‘replica’ of the regulatory module, in the form of a video (Supplementary Video 2); Fig. 4e shows 16 consecutive frames for 0 mM Ca$^{2+}$. Following the simulated time courses for three Ca$^{2+}$ states, individual RCK domains transition independently among $S_1$, $S_2$ and $S_3$ (represented by their respective crystal structures, in two views); Ca$^{2+}$ occupancy was determined as described above. This video is as effective way to exhibit the integrated model, which is fully defined by:

1. The kinetic model documented by the state diagram (Fig. 2c), its analytic solution (equation 5) and parameters (Supplementary Tables 1 and 2)
2. The crystal structural models of the three conformational states of RCK (PDB 1LNQ and 2FY8)
3. The state-specific orientations of helix $\alpha$B that directly link the corresponding states in the structural and kinetic models

**A spatiotemporal model for the whole MthK channel.** We previously predicted the Ca$^{2+}$-dependent $p_{bi}$ of the MthK channel from an energetic model, in which the regulatory module operates primarily in configuration ‘a’ under low Ca$^{2+}$ conditions and configuration ‘b’ under high Ca$^{2+}$ conditions (Supplementary Fig. 1). In both configurations, all RCK domains in the open species adopt $S_2$. However, in configuration a, the eight RCK domains can independently adopt any of the three conformations, yielding more than 6,000 closed species (without considering Ca$^{2+}$). A channel would thus spend the vast majority of time transitioning among closed species and rarely reach the open species. In contrast, in configuration b, each RCK
can adopt only $S_1$ or $S_2$ in the closed state, yielding merely a few hundred closed species; a channel should thus spend much less time in the closed state than it would in configuration $a$.

Raising the Ca\(^{2+}\) concentration would both reduce the lifetime of $S_1$, or $S_2$, and promote configuration $b$, therefore dramatically shortening the lifetime of the channel's closed state, in agreement with previous electrophysiological studies\(^9\). The lifetime of the channel's open state in the absence of Ca\(^{2+}\) is comparable to that of $S_0$ observed in the isolated regulatory module ($\tau_{\text{ch}} = 252 \pm 46$ ms (ref. \(^9\)) versus $\tau_{\text{ch}} = 241 \pm 12.7$ ms; Supplementary Table 3), whereas in near-saturating Ca\(^{2+}\) conditions it is comparable to the predicted lifetime for Ca\(^{2+}\)-bound $S_1$ in configuration $b$ ($\tau_{\text{ch}} = 2,030 \pm 332$ ms (ref. \(^9\)) versus $\tau_{\text{ch}} = 2,513 \pm 381/775$ ms; Supplementary Table 3 and Supplementary Note 5). If these lifetimes of $S_0$ numerically reflect those of the channel's open state, then raising the Ca\(^{2+}\) concentration should modestly lengthen the open channel's lifetime, also in agreement with previous electrophysiological studies\(^9\).

In the framework of the energetic model and on the basis of RCK's kinetics, we derived equation (8) (Box 1) and equation (9) (Box 2) to describe the Ca\(^{2+}\)-dependent lifetimes of the closed and open channel ($\tau_c$, and $\tau_o$), which are quantitatively checked below against those previously observed (Fig. 5a).

In equation (8) (Box 1), and equation (9) (Box 2), $K$ and $\text{Ca}K$ are defined in equation (10) (Box 3); $m$ is the number of RCK domains; $K$ is the apparent equilibrium constant of the channel gate; $k_o$ or $k_c$ is the reciprocal of the mean duration between the beginning of an $S_1$ event and the beginning of the next $S_1$ event, with or without Ca\(^{2+}\) bound ions, respectively (Supplementary Note 6, equation (47))); $k_c$ or $k_c^nK_0$ is the reciprocal of the mean duration between the end of an $S_2$ event to the beginning of the next $S_2$ event, with or without Ca\(^{2+}\) bound ions, respectively (Supplementary Note 6, underneath equation (48)); and the values of $n_i$ and $n_j$ specify the number of Ca\(^{2+}\) bound ions at distinct sites, among which the site denoted by $n_i$ may not necessarily be physically the same in configurations $a$ and $b$ (ref. \(^9\)).

The above expressions for $\text{obs} \tau_c$ and $\text{obs} \tau_o$ (equation (8) (Box 1) and equation (9) (Box 2)) lead to the equation that describes the dependence of the channel's $p_o$ on the Ca\(^{2+}\) concentration (equation 15 in ref. \(^9\)). The curves calculated using these equations predict the experimentally observed relation between $\text{obs} \tau_c$, $\text{obs} \tau_o$ (Fig. 5a) or $p_o$ (Fig. 5b) and the Ca\(^{2+}\) concentration within experimental errors\(^9\). Furthermore, we fitted equation 15 in ref. \(^9\), and rearranged versions of equation (8) (Box 1) and equation (9) (Box 2) (equations (56) and (57) in Supplementary Note 6) to the respective datasets. The fitted values, tabulated in Supplementary Table 1, were comparable to those used in the above calculations (Supplementary Tables 1–3), such that the fitted curves, if plotted, would be visually indistinguishable from the corresponding calculated curves.

We now have the essential information to assemble an integrated model for the whole channel, defined by:

(1) The kinetic model documented by the state diagram (Supplementary Fig. 1), its analytic solutions (equation (8) (Box 1) and equation (9) (Box 2)) and parameters (Supplementary Tables 1–3)

(2) The structural models documented by PDB 1K4C, 2FY8 and 3RBZ. Given that the model here requires at least two Ca\(^{2+}\)-binding sites per RCK, we used the PDB 3RBZ, in which Ca\(^{2+}\) ions bind each RCK domain in the open-state structure of the MhK channel at three sites\(^1\). A closed-state structure of the MhK pore is not yet available, and we thus used the closed-state structure of the pore domain from a different K\(^+\) channel\(^1\) (KcsA, PDB 1K4C) as a surrogate.

(3) The state-specific orientations of helix $\alpha B$ that directly link the corresponding states of RCK in the structural and kinetic models.

We performed simulations to obtain the temporal templates for the integrated channel model, which would in turn further validate our kinetic model. For each condition, the overall simulation consisted of eight concurrent simulations for the RCK domains and another one for the gate (Fig. 5c,d). For simplicity, the kinetics of the gate itself was assumed to follow the kinetics of the regulatory module during the transition between open and closed states. As
Fig. 5 | Comparison of the kinetic model with the observed data, and simulated kinetics and video frames for a whole channel. a, b. The open and closed lifetimes (a), as well as open probabilities (b), of the channel, calculated from simulated points (open symbols), are compared to previous experimentally observed data points (mean ± s.e.m.; closed symbols). The observed lifetimes \( \tau_a \) and \( \tau_c \) are so-called Ca\(^{2+}\)-dependent 'burst' and 'gap' times. The curves in a or b were calculated with equations (8) and (9) or equation (15) in ref. 1, respectively (see Supplementary Tables 1–3 for parameter values). \( K \) was set to a value of 1.7, and \( n_o \) to 1 (ref. 1); all parameters obtained from RCK were taken from Supplementary Tables 1–3. c. A crystal structure model is shown in a side view (upper) and a view along its central axis (lower), created as described in Fig. 4; the color-coding for RCK states is shown below. d. Concurrent simulations of state kinetics for eight RCK domains (A–H as assigned in e), where \( S_a \), \( S_b \), and \( S_c \) are color-coded in shades of yellow, blue and orange, respectively; the shade changes from a darker to a lighter one when the number of Ca\(^{2+}\) bound ions increases, as indicated on the right y axis. Configuration a or b, indicated on the left x axis, occupies the lower or upper half of each panel, separated by a dashed line. The residence of the gate in open (dark blue) and closed (light blue) states is shown in the bottom panel. e. Consecutive frames of a video segment of a whole channel model in the two views (Supplementary Video 3) generated as described in the text.

As defined by the model, the open state occurs when all RCK domains adopt \( S_a \). Also, as expected for a condition near EC\(_{50}\), the gate was open about half of the time. We calculated open and closed times, as well as open probabilities, from the simulated time courses of the gate's conformational changes and compared them, point-by-point, with the previous electrophysiology data\(^9\). The calculated values (open squares) matched those experimentally observed (closed circles) within experimental errors (Fig. 5a,b). Thus, as demonstrated both analytically and numerically, we have developed a model that satisfactorily accounts for the kinetics of the MthK channel.

We generated videos of a full channel molecule in a Ca\(^{2+}\) concentration near its EC\(_{50}\), featured in two views, along with the simulated time courses of individual RCKs and the channel gate (Supplementary Video 3); 16 consecutive frames from a short segment of the video are shown in Fig. 5e. The different behaviors of the molecule in three Ca\(^{2+}\) concentrations are compared in Supplementary Video 4. These videos allow the visualization of the integrated mechanistic model of the channel that quantitatively account for the channel's spatiotemporal characteristics at the resolution and accuracy levels of the underlying studies.

**Discussion**

We have resolved the events of a single RCK domain transitioning among three conformational states, by monitoring state-specific spatial orientations of an \( \alpha \)-helix attached with a fluorophore\(^4\). We then used the time course of the orientation changes as a temporal template to link the three structure states and create a video to exhibit a single RCK domain (Supplementary Video 1 and Fig. 1e). Next, from the state connectivity and lifetimes of individual conformational events of RCK, we determined 12 rate constants of conformational transitions, along with three \( K_0 \) values of Ca\(^{2+}\) binding (constrained with equilibrium analysis). These 15 constants fully

\[ \text{Open probability lifetimes (s)} \]

\[
\begin{array}{c|c|c|c}
\text{Ca\(^{2+}\) (mM)} & 0 & 0.1 & 10 \\
\hline
\text{Open probability} & 10^{-1} & 10^{-3} & 10^{-7} \\
\text{lifetimes (s)} & 10^{-5} & 10^{-1} & 10^{3} \\
\end{array}
\]
established a six-state model that is minimally required to quantitatively account for RCK’s multi-state kinetic behaviors. Furthermore, in the framework of an energetic model of the MthK channel, we deduced, from the kinetic characteristics of RCK, a model that quantitatively predicts the channel’s kinetics within experimental errors. This predictability serves as a validation of not only this model but also our experimental approach. Despite working with the isolated regulatory module, introducing cysteine mutations, attaching the fluorophore and anchoring the protein to a surface, the information necessary for deducing the kinetics of the whole channel was meaningfully preserved.

Finally, on the basis of state-specific spatial characteristics, we linked the present kinetic model and the available structural models together, to create an integrated spatiotemporal model of a whole channel. Thus, a combination of the present method and existing structural techniques, which are based on the efforts of numerous scientists, has now created an effective integrative version of dynamic structural biology, which should be applicable to the study of a wide range of proteins.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41594-019-0276-0.

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Author contributions
J.H.L. and Z.L. designed the study. J.H.L. performed experiments, developed analytical tools, and analyzed the data, with the input from Z.L. J.H.L. and Z.L. interpreted the results and wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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Methods
Protein sample preparation and data collection. As described in ref. 4, a recombinant protein of the MthK channel’s regulatory module, which contained an N-terminal recognition sequence for biotin ligase, a C-terminal His-tag with a preceding specific protease-cutting sequence, and the double E146C and L153C mutation in helix αB, was produced using the bacterial BL-21 expression system. The protein was labeled with bifunctional rhodamine (bis-((N-iodoacetyl)-piperazinyl)-sulfonerhodamine; Invitrogen B10621) via the two mutant cysteine residues and attached to a coverslip conjugated with streptavidin (Arrayit) via biotinylated N termini. Polarized emissions from individual bifunctional rhodamine labels, excited in the evanescent field created at the surface of the sample coverslip by a circularly polarized laser beam (532 nm), were collected via a fluorescence microscope with four polarization emission channels onto an electron-multiplying charge-coupled device camera, while the sample protein was immersed in a solution containing 200 mM KCl, Ca^{2+} of various concentrations and 10 mM HEPES titrated to pH 8.0, where 1 mM EGTA was used as a buffer in the nominal Ca^{2+}-free and low (0.1 mM) Ca^{2+} solutions.

Data collection and analysis. Also as described in ref. 4, each intensity of the four emission components collected from a given fluorophore was a direct summation of individual pixels. I_{θ}, I_{φ} and I_{Ω} were calculated using equations (62), (63), (61) and (70) in the Supplementary Notes 3 and 6 in ref. 4, respectively. Conformational transitions and states were identified in two separate steps. A changepoint algorithm was applied to the intensity traces to detect the transitions between conformational events, whereas a k-means-cluster-based algorithm was applied to identify the conformational states of individual events based on both θ and φ.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Data and materials described here will be made available upon reasonable request.
Reporting Summary

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|     | • Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated |
|     | • Clearly defined error bars |
|     | • State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

Data collected using an emccd camera in conjunction with NIS elements from Nikon.

Data analysis

For each fluorescent point source, the time-dependent intensities were calculated using a custom Imagej plugin written in Java. From these intensities, angles were calculated using a separate program written in Labview. This same program also performs event detection and determines which of three states each event belongs to. Other programs, also written in Labview, were used for viewing the results of this analysis. Algorithms underlying these programs are described in the methods section in reference 12. The programs themselves can be made available upon request.

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Raw data can be found in Figs. 3 and 5 of reference 5 and Fig. 1 of reference 4. Raw data can be made available on request, but is not currently publicly available.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

Each distribution described in the paper and shown in figures was formed from events of observed individual MthK regulatory domains under different calcium conditions. Over all seven calcium conditions, ~1,000 total events were recorded. Within each condition, separate distributions were formed for each of the three conformational states. This ensures on average about 500 events per distribution. Sample sizes were not predetermined. However, each normal distribution is symmetrical and well defined with standard deviations around 7 degrees, giving standard errors of around 0.3 degrees.

Data exclusions

Data were excluded on the following basis: 1) The model for polarized fluorescence has the expectation that although individual polarized intensities can vary over time, the total combined intensity should not. Therefore, any traces that showed any significant time dependent changes in the total intensity were excluded. 2) For a given recording, at least 10 events are required to obtain a 95% confidence level for state identification, so any trace with less is excluded. 3) For event detection and state identification, a signal to noise ratio greater than 4 is required for a 95% confidence level - traces with less are excluded. 4) States 1, 2 and 3 were identified according to theta - some traces were resolved only in phi and not in theta, and so were excluded from analysis.

Replication

These experiments were performed over 3 separate days. Each day’s experiments had comparable results.

Randomization

Randomization as is described here is not relevant. Because randomization is inherent to the process being observed (i.e. stochastic movement of a single protein) it is not under our control. This is a physical study of the response of a single type of protein (MthK) to a single type of ligand (calcium) of varying concentrations.

Blinding

Blinding is not relevant for the same reason that randomization is not relevant.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a

☑️ Unique biological materials
☐ Antibodies
☐ Eukaryotic cell lines
☐ Palaeontology
☐ Animals and other organisms
☐ Human research participants

Methods

n/a

☑️ Involved in the study
☐ ChiP-seq
☐ Flow cytometry
☐ MRI based neuroimaging