Energetics of ångström-scale conformational changes in an RCK domain of the MthK K⁺ channel

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Allosteric proteins transition among different conformational states in a ligand-dependent manner. Upon resolution of a protein’s individual states, one can determine the probabilities of these states, thereby dissecting the energetic mechanisms underlying their conformational changes. Here we examine individual regulator of conductance to K⁺ (RCK) domains that form the regulatory module of the Ca²⁺-activated MthK channel. Each domain adopts multiple conformational states differing on an ångström scale. The probabilities of these different states of the domain, assessed in different Ca²⁺ concentrations, allowed us to fully determine a six-state model that is minimally required to account for the energetic characteristics of the Ca²⁺-dependent conformational changes of an RCK domain. From the energetics of this domain, we deduced, in the framework of statistical mechanics, an analytic model that quantitatively predicts the experimentally observed Ca²⁺ dependence of the channel’s open probability.

The Ca²⁺-activated MthK channel consists of a transmembrane pore module and a cytoplasmic regulatory module. The regulatory module is formed by eight RCK domains. Three conformational states, S₁, S₂, and S₃ (refs. ¹; PDB 1LNQ, PDB 2FY8 and PDB 4RO0) of the RCK domain have been captured via crystallography (all notations and abbreviations are listed in Supplementary Note 1). Initially, one Ca²⁺-binding site was identified in each RCK domain, and a subsequent study revealed two additional sites (Supplementary Fig. 1). Thus, a total of 24 Ca²⁺-binding sites are present in the regulatory module. Electrophysiology studies have shown that Ca²⁺ binding to the regulatory module increases the channel’s open probability (pₒ) by approximately four orders of magnitude, with a measured Hill coefficient as high as ~20 and an average of ~10 (refs. ², ³). These behaviors could be explained using a modified version of the Monod–Wyman–Changeux (MWC) model, which involves a series of cooperative binding of numerous Ca²⁺ ions (modified MWC models were also used in earlier studies of other types of channels⁴, ⁵). To a large degree, the apparent strong, cooperative Ca²⁺ binding reflects cooperative interactions among RCK domains during channel activation.

To understand the gating mechanism of the MthK channel, it is necessary to directly examine the regulatory module itself, a task that cannot be accomplished with single-channel current-recording techniques. Here, we use a polarization microscope–based method to investigate the Ca²⁺-dependent conformational changes of a single, fluorescently labeled RCK domain within an isolated regulatory module of the MthK channel. Based on the probabilities of individual conformational states determined here, we set out to establish a model that quantitatively accounts for the Ca²⁺-dependent regulation of RCK conformational changes and to obtain, in the framework of statistical mechanics, an analytic solution that can predict the observed pₒ of the channel over a wide range of Ca²⁺ concentrations.

Results

Ca²⁺-dependent conformational changes in RCK. As described in the accompanying study, we examined the spatial orientation changes of helix αB in the RCK domain, which is located closest to the channel’s gate and adopts a unique orientation in each of the three conformational states identified via crystallography. In one of the eight RCK domains within the regulatory module, this α-helix was labeled with a bifunctional rhodamine molecule via two mutant cysteine residues. Individually labeled regulatory modules (without the pore module) were attached to a coverslip via a four-fold attachment, so the central axis would be aligned with the optical (z) axis. We collected fluorescence intensities from individual particles via four polarization channels in different Ca²⁺ concentrations (Fig. 1a, b), from which we calculated the total emitted intensity (Iₒ), and inclination (θ) and rotation (φ) angles of the fluorophore’s dipole and thus of the α-helix (Fig. 1c, d). We also calculated the angle change between two conformations in the actual rotation plane (Ω), which is a function of θ and φ. The state S₃ with an intermediate θ value, was chosen as a reference; consequently, Ω₃ values were distributed around zero. The black traces superimposed on the experimental intensity and calculated angle traces were generated by setting the amplitude of a given event uniformly to the average of the observed values within that event, a procedure that increased the effective signal-to-noise ratio and thus the angle resolution of individual events. Starting and end points of individual events were statistically determined from the concurrent changes in all four intensity traces.

We identified individual conformational states on a particle-by-particle basis, to eliminate the interparticle variability, and on the basis of its θ and φ angles, to increase resolution and reliability. As θ is unique for each of the three crystallographically identified conformational states, we used it to identify the corresponding states. To achieve greater accuracy, we estimated the θ value of each state from the distribution of mean θ values of individual particles analyzed separately (Fig. 2a). For each of the three states, the values of either θ or Ω were similar across all examined Ca²⁺ concentrations. Furthermore, across these concentrations, the mean θ values and the mean Ω values of the three states were comparable to those values predicted from the crystal structures (Fig. 2b).

Ca²⁺ dependence of state probabilities of RCK. We built distributions of the three states in various Ca²⁺ concentrations from the
implies the existence of a minimum of six states: three conformations without Ca$^{2+}$ (S$_1$, S$_2$, and S$_3$) and three with $n_i$ number of Ca$^{2+}$ bound (S$_1$, Ca$_{n_i}^{2+}$, S$_2$, Ca$_{n_i}^{2+}$, and S$_3$, Ca$_{n_i}^{2+}$). Because all RCK domains adopt S$_2$ in the open-state structure, we chose this state as a reference for other states to determine the following equilibrium constants:

$$K_{i,2} = \frac{[S_i]}{[S_2]}; \quad K_{n_i} = \frac{[S_i, Ca_{n_i}^{2+}]}{[S_i, Ca_{n_i}^{2+}]; \quad K_{Di} = \frac{[S_i][Ca_{n_i}^{2+}]}{[S_i, Ca_{n_i}^{2+}]; \quad i = 1, 2, 3 \quad (1)}$$

$K_{i,2}$ and $K_{n_i}$ could be calculated directly from the state populations under zero- and saturating Ca$^{2+}$ conditions, respectively, and $K_{Di}$ could be independently determined from the ‘midpoint’ positions of the population curves (Fig. 3b). Together, these constants would fully define the model quantitatively.

The Ca$^{2+}$ dependence of the probability ($p_i$) of a labeled RCK domain to adopt a given conformation $i$ should reveal its relationship with other RCK domains within the regulatory module. If RCK domains were independent of each other and Ca$^{2+}$ effectively bound to only one site in an RCK domain, the Ca$^{2+}$ dependence of $p_i$ would follow an equation describing the Ca$^{2+}$ and RCK interaction in a one-to-one stoichiometry. However, if there were a high degree of positive cooperativity among Ca$^{2+}$ sites located within an RCK or among RCK domains, as is the case with the full channel, the exponent $n_i$ of the Ca$^{2+}$-concentration term in equation (1) would be much greater than 1.

The observed concentration of RCK in all six conformational states [obs$S_i$], three without and three with $n_i$ of Ca$^{2+}$ bound, is given by:

$$[\text{obs}S_i] = [S_1] + [S_2] + [S_3] + [S_1, Ca_{n_i}^{2+}] + [S_2, Ca_{n_i}^{2+}] + [S_3, Ca_{n_i}^{2+}] \quad (2)$$

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Under an equilibrium condition, only five out of the nine equilibrium constants (in certain combinations) would be independent parameters in the model (equation (1) and Fig. 3c). We chose to equilibrate the channel’s open state. The observations that all eight RCK domains adopt the conformation underlying the open state should be related to the probability that individual RCK domains adopt the conformation underlying the channel’s open state. The observations that all eight RCK domains adopt the conformation underlying the open state should be related to the probability that individual RCK domains adopt the conformation underlying the channel’s open state (Fig. 3b) are consistent with the notion that all RCK domains primarily adopt $S_2$ (dubbed all-$S_2$) in the open state. Furthermore, the probability of all-$S_2$ in the absence of Ca$^{2+}$, given by $p_{S_2} = 5.1 \times 10^{-4}$, is comparable to the experimentally determined spontaneous $p_d(2.6 \times 10^{-4})$ of the MthK channel (Supplementary Table 2).

We fit equation (3) globally to the three relations between state probabilities and Ca$^{2+}$ concentrations (Fig. 3b). The resulting values of all equilibrium constants are presented in Supplementary Table 1. The fitted $n_i$ value was 0.64, consistent with the mechanism that Ca$^{2+}$ binding primarily to one site in individual RCK domains promotes the Ca$^{2+}$-dependent redistribution among the three conformational states. The observed value of $<1$ may reflect errors of measurements or a modest negative cooperative interaction from another site. In any case, the shallow Ca$^{2+}$ dependence indicates that, in the isolated regulatory module, RCK domains did not exhibit the level of positive cooperativity expected from the steep Ca$^{2+}$ dependence of MthK channel activation and appeared to undergo the observed conformational changes largely independently.

If individual RCK domains undergo conformational changes independently, then the spontaneous $p_d$ of a MthK channel under Ca$^{2+}$-free conditions should be related to the probability that individual RCK domains adopt the conformation underlying the channel’s open state. The observations that all eight RCK domains adopt $S_2$ (dubbed all-$S_2$) in the open state. Furthermore, the probability of all-$S_2$ in the absence of Ca$^{2+}$, given by $p_{S_2} = 5.1 \times 10^{-4}$, is comparable to the experimentally determined spontaneous $p_d(2.6 \times 10^{-4})$ of the MthK channel (Supplementary Table 2).
Deducing an energetic model of the MthK channel. If the RCK domain acts as the basic functional unit of the regulatory module, then establishing an energetic model for the whole MthK channel only requires further deducing the energetics of the gate plus those of additional unobserved configurations and Ca\(^{2+}\)-RCK interactions that occur in the regulatory module of a whole channel. Below, we develop a model to quantitatively predict the energetic hallmark of regulation of the MthK channel by Ca\(^{2+}\), in the form of the \(p_o - [\text{Ca}^{2+}]\) relationship. Detailed statistical mechanics-based derivations are presented in Supplementary Notes 2–4.

In an isolated regulatory module, eight independent RCK domains can individually adopt three conformations, giving rise to \(3^8 = 6,561\) possible permutations. Of these 6,561 permutations, if only the all-S2 species underlies the open state of the regulatory module (RMo), then the remaining permutations would represent a closed-state configuration (a), denoted by aRMc (Fig. 4a). As such, individual RCK domains in aRMc would independently adopt any conformations, with or without Ca\(^{2+}\) bound. The concentration of aRMc would be given by:

\[
[\text{aRM}_c] = \left[\text{S}_2\right] \times \left[\text{S}_3\right] \times \left[\text{Ca}^{2+}\right]_8 \tag{5}
\]

where \([\text{aaS}]\) is as defined in equation (2); and \(m\) is 8, the number of the RCK domain.

We also considered a previously proposed configuration (b) of the closed state, denoted by bRMc, in which individual RCK domains can adopt S1 or S3 (ref. 13). Thus, in our model, during the transition from aRMc to bRMc, S2 would be excluded from all closed species (Fig. 4b). The coexistence of Ca\(^{2+}\)-free S1 and S3 has been observed crystallographically (ref. 2). We further stipulate that bRMc would exist with or without Ca\(^{2+}\) bound (ref. 7). Without explicitly invoking any additional distinct intermediate species, a transition from aRMo (open state, all RCK domains in S2) to bRMc (closed state, S2 excluded) would be perceived as a cooperative transition, because all eight RCK domains must transition to S1 or S3. As such, we modeled the binding of Ca\(^{2+}\) to individual RCK domains in bRM as being cooperative (Fig. 4b). The concentration of bRMc should then be expressed as:

\[
[\text{bRM}_c] = \left[\text{S}_2\right] \times \left[\text{S}_3\right] \times \left[\text{Ca}^{2+}\right]_8 \tag{6}
\]

Regarding the open state, we treated the all-S2 species as the open state (RMo). The observed concentration of all-S2 species in configuration \(\text{RM}_m\) or \(\text{RM}_c\) (with or without Ca\(^{2+}\) bound) is given by equation (7) or equation (8).

\[
[\text{obs}[\text{RM}_m] = \left[\text{S}_2\right] + \left[\text{S}_2 \cdot \text{Ca}^{2+}\right]_8 \tag{7}
\]

\[
[\text{obs}[\text{RM}_c] = \left[\text{S}_2\right] + \left[\text{S}_2 \cdot \text{Ca}^{2+}\right]_8 \tag{8}
\]
Given that \( \text{RM} \) would have far fewer states than \( \text{RM} \), it should have higher energy. Implicitly, the binding of Ca\(^{2+} \) to at least one additional site (denoted by \( n_1 \)) in individual RCK domains would be required to lower the free energy of \( \text{RM} \). Operationally, Ca\(^{2+} \) binding to the \( n_1 \) site would regulate the relative distribution between \( \text{RM} \) and \( \text{RM} \), whereas Ca\(^{2+} \) binding to the \( n_1 \) site would regulate the relative distribution among the RCK conformations. However, what is denoted by \( n_1 \) may not necessarily be the same physical site in \( \text{RM} \) and \( \text{RM} \). Below, we examine first the \( p_o \) predicted on the basis of \( \text{RM} \) or \( \text{RM} \) alone, and then the energetic characteristics of the \( n_1 \) site. Substituting equations (4–8) into the following defining relation:

\[
p_o = \frac{[O]}{[O] + [C]}
\]

where \([O]\) and \([C]\) denote the concentrations of the open and closed states, we obtained the expressions for the regulatory module’s \( p_o \) in \( \text{RM} \) and \( \text{RM} \):

\[
\text{Observed } p_o = \frac{[\text{obs}_{\text{RM}O}] + [\text{obs}_{\text{RM}O}]}{[\text{obs}_{\text{RM}C}] + [\text{obs}_{\text{RM}C}]} = \frac{(1 + |\text{Ca}^{2+}|^n_{\text{Kap}})^m}{K + 1 + (\text{Ca}2^+)^n_{\text{Kap}}}
\]

Substituting equations (13) and (14) (in the form of energetic constants) into equation (9) yields equation (15) (Box 1) for describing the \( p_o = |\text{Ca}^{2+}| \) relation for the whole channel.

In equation (15) (Box 1), \( K \) is the apparent equilibrium constant of the gate, as defined in Supplementary Note 4; \( K \) describes the relative distribution between \([S_1] + [S_2]\) (unbound closed species in configuration b) and \([S_1] \) (open species); and \( |\text{Ca}^{2+}| \) describes the relative distribution between the corresponding Ca\(^{2+}\)-bound closed and open species (equation (4)). In an ideal case in which the total amount of energy related to this fully reversible conformation redistribution equals that derived from Ca\(^{2+}\) binding, \( K \) should equal \( |\text{Ca}^{2+}| \). We noted that fitted \( K \) (1.576) is slightly smaller than \( |\text{Ca}^{2+}| \) (1.686) (Supplementary Table 1). The small ‘excess’ Ca\(^{2+}\)-binding energy defined by \( kT \ln (K^{\text{Ca}^{2+}}^{−1}) \) could, in principle, help to energetically equalize the open and closed states of the channel gate itself. \( (K^{\text{Ca}^{2+}})^{−1/2} \) yields a value of 1.3; within experimental errors, a \( K \) of 1.3 (slightly favoring the closed state) would be sufficient to close the small gap between the observed minimal \( p_o \) and that predicted by the model for the regulatory module alone (that is, equation (15) (Box 1) where \( K \) is set to 1).

To test the predictability of our model, we calculated a \( p_o = |\text{Ca}^{2+}| \) curve with equation (15) (Box 1), using the parameters determined from fluorescence polarization measurements (Fig. 3c and Supplementary Table 1; \( K = 1.3, K_{\text{N2A}} = 0.38 \text{ mM}, \) and \( n_1 = 1 \)). The calculated curve matches the \( p_o = |\text{Ca}^{2+}| \) relationship previously determined via electrophysiology, within the experimental errors.
(Fig. 5b). It is noteworthy that when explicitly expressed, equation (15) (Box 1) would be fully defined by a total of ten parameters: $K_{1,2}$, $K_{3,2}$ (expressed together as $K_{K}$), $Ca_{K}$, $n_1$ and $K_{Dn2}$, all determined from studying RCK; $m$ as determined via crystallography; $n_2$, $K_{Dn2}$ and $g_{K}$ can be deduced as described above. This large number of parameters cannot be extracted by fitting the $p_o - [Ca^{2+}]$ curve with a Hill equation.

For comparison, we estimated $g_{K}$ and $K_{Dn2}$ by fully constraining them with experimental measurements. First, we used equation (59) (Supplementary Note 4) to directly calculate $g_{K}$ from the constant $K$ (equation (4) and Supplementary Table 1) and minimal $a_p$ of the channel (Supplementary Table 2), obtaining a slightly larger value of 1.7. We then determined $K_{Dn2}$ through a global fit of equation (15) (Box 1) to the experimental $p_o - [Ca^{2+}]$ relationship of the channel (Fig. 5b) and the three plots regarding $Ca^{2+}$ dependence of RCK conformations in the isolated regulatory module (Fig. 3b; all resulting parameters are tabulated in Supplementary Table 1). The fit yielded a $K_{Dn2}$ value of 0.39 mM, nearly the same as the deduced value of 0.38 mM. In the fit, $g_{K}$ was set at 1.7, instead of 1.3, and, consequently, the calculated and fitted curves diverged slightly and could be recognized. We infer that given the rather small $g_{K}$, the observed channel’s operational energy primarily reflects that of the regulatory module and that the present model (equation (15) (Box 1); Fig. 5) quantitatively predicts the experimental $p_o - [Ca^{2+}]$ relationship of MthK.

**Discussion**

The regulatory module of the MthK channel harvests the so-called gating energy from the binding of $Ca^{2+}$ to its eight RCK domains. In the model described here, each RCK in RM may independently adopt three conformations, with or without $Ca^{2+}$ bound. With only a single $Ca^{2+}$ site per RCK considered, eight independent
RCM domains could generate 6^6 (~1.7 million) possible permutations, or 3^6 (6,561) permutations in the absence of Ca\(^{2+}\). If among this number of permutations, only all-\(S\) is associated with the open conformation of the channel, the spontaneous \(p_o\) would be primarily determined by entropic energy, given the vastly different numbers of the accessible open versus closed species. The large number of closed species should be depopulated toward the limiting case of effectively one state by Ca\(^{2+}\) binding, and the collective probability of all closed species in saturating Ca\(^{2+}\) concentrations should be much smaller than the probability of the open species, such that the channel’s \(p_o\) would rise from the minimum toward 1. Indeed, electrophysiology studies have shown that Ca\(^{2+}\) regulation of the MthK channel occurs primarily while the gate is closed\(^{4,5}\).

A proposal that a MthK channel can open from the all-\(S\) state has been put forward, on the basis of the following observations\(^{6}\). First, an all-\(S\) form of the isolated regulatory module was captured crystallographically, with Ba\(^{2+}\) present at multiple sites within individual RCM domains. Second, Ba\(^{2+}\) can activate the MthK channel, albeit with lower efficacy and potency than Ca\(^{2+}\). Third, a mutation at a putative Ba\(^{2+}\) binding site eliminates the ability of Ba\(^{2+}\) to activate the channel. Thus far, there is no structural evidence to link all-\(S\) to the open state of the channel. In any case, the probability of all-\(S\) species predicted from our measurements is low, and thus, the \(p_o = \text{[Ca}^{2+}\] \) curves calculated from two compared models, in which either all-\(S\) only or all-\(S\) and all-\(S\) are open species, are visually indistinguishable, even on double logarithmic plots (Supplementary Fig. 2). Should Ba\(^{2+}\) act differently than Ca\(^{2+}\), such that Ba\(^{2+}\) binding to RCK would populate \(S\) instead of \(S\), modeling all-\(S\) species as an open state could in principle accommodate the observed channel activation by Ba\(^{2+}\).

Here we considered only all-\(S\) as the open species for the following reasons. First, only all-\(S\) was observed in an open-state structure of the MthK channel. Second, we intended to account for the Ca\(^{2+}\)-dependent regulation, for which all-\(S\) being the open species is sufficient. Third, we do not have the quantitative information regarding Ba\(^{2+}\)-bound RCK to express the all-\(S\) species in the analytic model. In any case, the present model could be expanded to include additional open and closed species to account for other features of the channel.

As mentioned, one defining feature of the MWC model is that a protein may adopt one of two (or more) alternative states (R or T) with or without ligand bound\(^7\). A second defining feature is that all protomers in the protein must be in the same state. In our model, ~1.7 million species form a grand canonical ensemble in the limiting case of effectively one state by Ca\(^{2+}\) binding, and the collective probability of all closed species in saturating Ca\(^{2+}\) concentrations is low, and thus, the probability of individual RCMs to adopt \(S\) and thereby-fine tuning \(p_o\). Second, cooperative Ca\(^{2+}\) binding would populate \(RM\) over \(RM\), of which the former has fewer closed states. These tuning and configuration-switching mechanisms primarily underlie the shallow and steep phases of the \(p_o = \text{[Ca}^{2+}\] \) relationship, respectively. The good agreement between our data obtained with the isolated regulatory module and previous electrophysiology data of the whole channel validates our approach. The present energetic study serves as a foundation for a subsequent study\(^8\), in which we use the present method to examine the multistate conformational dynamics of RCK and demonstrate how the resulting dynamic information can be used as a temporal template to link its existing structures.

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/s41594-019-0275-1.

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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.
Methods

Sample preparation and data recording. As described in ref. 12, a recombinant protein of the MthK channel regulatory module containing 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)-Sulfonyl rhodamine; 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; the sample protein was immersed in a solution containing 200 mM KCl, various [Ca\textsuperscript{2+}], and 10 mM HEPES titrated to pH 8.0, in which 1 mM EGTA was used as a buffer in the nominal Ca\textsuperscript{2+}-free and low-Ca\textsuperscript{2+} (0.1 mM) solutions.

Data analysis. Data analysis is also as described in ref. 12. Each intensity of the four emission components collected from a given fluorophore was a direct summation of individual pixels. I<sub>θ</sub>, I<sub>φ</sub>, I<sub>Ω</sub>, and I<sub>φ</sub> were calculated using equations 62, 63, 61 and 70 in Supplementary Notes 3 and 6 of ref. 12, 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.

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Data availability

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

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research **guidelines for submitting code & software** for further information.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Raw data can be found in Fig. 1. Raw data can be made available on request, but is not currently publicly available.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences
☐ Behavioural & social sciences
☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/reportingSummary-flat.pdf

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, ~10,000 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 symmetric 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 Involved in the study

☐ 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