Role of Dynamics in the Autoinhibition and Activation of the Exchange Protein Directly Activated by Cyclic AMP (EPAC)*

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The exchange protein directly activated by cAMP (EPAC) is a key receptor of cAMP in eukaryotes and controls critical signaling pathways. Currently, no residue resolution information is available on the full-length EPAC dynamics, which are known to be pivotal determinants of allosteric processes. In addition, no information is presently available on the intermediates for the classical induced fit and conformational selection activation pathways. Here these questions are addressed through molecular dynamics simulations on five key states along the thermodynamic cycle for the EPAC-dependent activation of a fully functional construct of EPAC2, which includes the cAMP-binding domain and the integral catalytic region. The simulations are not only validated by the agreement with the experimental trends in EPAC-binding domain dynamics determined by NMR, but they also reveal unanticipated dynamic attributes, rationalizing previously unexplained aspects of EPAC activation and autoinhibition. Specifically, the simulations show that EPAC binding causes an extensive perturbation of dynamics in the distal catalytic region, assisting the recognition of the Rap1b substrate. In addition, analysis of the activation intermediates points to a possible hybrid mechanism of EPAC allosteric incorporating elements of both the induced fit and conformational selection models. In this mechanism an entropy compensation strategy results in a low free-energy pathway of activation. Furthermore, the simulations indicate that the autoinhibitory interactions of EPAC are more dynamic than previously anticipated, leading to a revised model of autoinhibition in which dynamics fine tune the stability of the autoinhibited state, optimally sensitizing it to EPAC while avoiding constitutive activation.

The exchange protein directly activated by cyclic AMP (EPAC) is a major protein involved in intracellular G-protein-mediated signaling in eukaryotic organisms, playing a regulatory role in such processes as cell adhesion, cell-cell junction formation, and insulin secretion by pancreatic β-cells (1–10). Two isoforms of EPAC have been identified, EPAC1 and EPAC2, both composed of an amino-terminal regulatory region (RR), and a carboxyl-terminal catalytic region (CR; Fig. 1a) (11, 12). The regulatory region consists of a Dishevelled Egl-10 Pleckstrin (DEP) domain (13) and one (for EPAC1) or two (for EPAC2) cyclic nucleotide-binding (CNB) domains (Figs. 1 and supplemental Fig. S1) (11, 12, 14, 15). The catalytic region consists of the CDC25-homology domain (CDC25-HD) responsible for guanine nucleotide exchange activity, as well as a Ras exchange motif (REM) domain and a Ras association (RA) domain involved in the subcellular localization of EPAC (Fig. 1a) (11, 12, 14, 15).

The structures of EPAC solved in the absence and presence of a nonhydrolysable agonist closely related to cAMP have shown that EPAC adopts two main conformations, which differ with respect to the relative RR/CR orientation (Fig. 1b) (14, 15). In the so-called “closed” conformation the RR is in the vicinity of the catalytic domain, blocking access to the Rap1b substrate and effectively inhibiting the guanine nucleotide exchange activity (14). In the so-called “open” conformation a hinge helix rotation displaces the RR away from the CR, making the catalytic site accessible to the substrate and leading to activation (Fig. 1b) (15). In the absence of cAMP, EPAC populates mainly the closed inactive conformation, which is stabilized by two key sets of interactions commonly referred to as the hydrophobic hinge (HH) and ionic latch (IL) (14–17). The HH involves the phosphate binding cassette (PBC), which has been proposed to sterically block conformational changes in the hinge helix, maintaining this helix in its inactive conformation (supplemental Fig. S1). Specifically, it has been hypothesized that the inactive conformation is stabilized by steric hindrance between a leucine side chain from the PBC (Leu808 in EPAC2) and a phenylalanine side chain from the hinge (Phe455 in EPAC2) (14, 16). The IL involves the CNB and CDC25-HD domains, which interact through a series of key salt bridges between an N-terminal helical bundle (NTHB) in the CNB domain and the catalytic site of the CDC25-HD domain (14). In particular, these IL interactions involve the side chains of a glutamine (Gln303 in EPAC2) and aspartate (Asp307 in EPAC2) residue from the
DEP-CNB connecting helix ("α1" of the NTHB), a glutamate residue (Glu332 in EPAC2) from the second NTHB helix ("α2"), and an aspartate (Asp883 in EPAC2) and arginine (Arg886 in EPAC2) residue from the CDC25-HD domain catalytic site (Fig. 1b) (14).

When cAMP binds to the CNB domain, a number of changes occur that perturb the HH and IL autoinhibitory interactions and allow EPAC to adopt its active conformation. First, the interaction of the bound cAMP with the PBC repositions Leu408, relieving the steric hindrance between the PBC and the hinge helix and allowing the activation-associated hinge rotation to proceed (15, 16). The second major change induced by cAMP binding is a breaking of the ionic latch interactions between the NTHB of the CNB domain and the catalytic site of the CDC25-HD, assisting the displacement of the EPAC regulatory region away from the CDC25-HD via the hinge rotation (14, 15). Notably, NMR relaxation experiments have shown that upon cAMP binding, an enhancement of conformational dynamics occurs in the NTHB, including the second and third residues involved in the ionic latch (Asp307 and Glu332 in EPAC2), whereas no major structural changes appeared to occur in the helices that span the ionic latch (i.e. H92511 and H92512) (18). Therefore, cAMP binding triggers an increase in conformational entropy of the ionic latch region, thereby increasing the entropic penalty for preserving the ionic latch, and consequently weakening the ionic latch-mediated interactions between the CNB and CDC25-HD domains (18).

Besides causing the HH rearrangement and IL weakening, cAMP forms through its adenine additional interactions with the base binding region (BBR) of the CNB and the so-called lid...
region (supplemental Fig. S1), thereby stabilizing EPAC into its active conformation (15, 16). The active structure of EPAC is further stabilized by intramolecular interactions that are formed among the CNB and REM domains and the lid, including interactions of PBC residue Lys405 with lid residues Glu443, Asn445, and Tyr480, and interactions of CNB residues Gln369 and Tyr551 with REM residue Tyr551 (15). Overall, it is clear that the experimental characterizations of the apo/inactive and holo/active states of EPAC have unveiled key attributes of the mechanisms of EPAC autoinhibition and cAMP-dependent activation (14–21). However, several critical questions remain open. For instance, whereas NMR has shown that modulations of dynamics by cAMP represent a pivotal feature of EPAC activation and cyclic nucleotide selectivity (16, 18, 22, 23), the NMR studies have been confined only to the CNB domain. Although dynamics elsewhere in the protein are expected to be critical for allosteric/conformational selection mechanism. 

For the purpose of gaining insight into the dynamics of full-length EPAC in all four states of the thermodynamic cycle of EPAC activation (Fig. 1b), we present here molecular dynamics (MD) simulations (34–38) on a functional EPAC2 construct that includes both the regulatory CNB and the integral catalytic region. The simulations are in agreement with the available NMR data, but they also reveal several picosecond-nanosecond dynamic features of EPAC allostery that were not anticipated from previous experiments. These features include a dynamic control of critical autoinhibitory interactions as well as an extensive modulation of both RR and CR dynamics by both cAMP and Rap1b binding, pointing to the existence of a long-range dynamic RR/CR cross-talk. In addition, the MD simulations reveal a possible low free-energy pathway for the reversibility and cAMP-dependent activation of EPAC through a hybrid induced-fit/conformational selection mechanism.

**EXPERIMENTAL PROCEDURES**

MD simulations in explicit solvent were performed starting from the four states of the thermodynamic cycle for the cAMP-dependent EPAC allostery (Fig. 1b), i.e. the experimentally observable apo/inactive and holo/active states as well as the intermediate holo/inactive and apo/active states (18). The latter two states are unstable under physiological conditions and are referred to herein as metastates to distinguish them from the experimentally observable apo/inactive and holo/active states, for which structures have been solved by x-ray crystallography (14, 15). Furthermore, an additional MD trajectory was generated starting from the holo/inactive state bound to the Rap1b substrate. All simulations were performed on the EPAC2(280–990) fragment (Fig. 1b). This segment contains the essential regulatory CNB domain and the full catalytic region (i.e. the REM, RA, and CDC25-HD domains) (14, 15). The EPAC2(280–990) construct was previously shown to be fully functional as it exhibited cAMP-dependent guanine nucleotide exchange factor activity comparable with that of wild-type EPAC2 (14).

A summary of the MD simulations performed for the three states and the two metastates of EPAC2(280–990) is given under supplemental Table S1. The structural dynamics during the last 50 ns of each MD trajectory were examined through multiple and complementary analyses, including root mean square deviations (r.m.s. deviations; Fig. 2), contact distance time profiles (supplemental Figs. S4 and S7), backbone N-H order parameters (S2; Figs. 3 and 4), dynamic cross-correlation matrices (DCCMs; Fig. 5), differential root mean square fluctuations (Figs. 6 and supplemental S3), covariance matrices (supplemental Fig. S5), and principal component analyses (PCA; supplemental Fig. S6).

Each type of analysis examined key aspects of EPAC dynamics: r.m.s. deviations from the initial structures were performed to probe the stability of the overall domain topology of EPAC (i.e. closed versus open; Fig. 1b); contact distance time profiles were utilized to assess the dynamics of key inter-domain interactions that selectively stabilize either the open or closed topology; and backbone N-H order parameters (S2) were used to probe the amplitudes of local dynamics at residue resolution. Local dynamics are in principle also probed by r.m.s. fluctuations, provided that the structural superimposition required by r.m.s. fluctuation analysis is confined to a single domain to minimize contributions from long-range interdomain motions. The resulting domain-specific r.m.s. fluctuation profiles provide a picture of the amplitudes of local fluctuations that is qualitatively similar to the S2 plots (supplemental Fig. S2), but the S2 analysis benefits from the added advantage of facilitating validation of the MD results against the experimental NMR data available for the CNB domain (18). Further details about the S2 versus r.m.s. fluctuation comparisons are available as supplemental data. Correlations between dynamic fluctuations at different locations, either intra- or interdomain, were quantified through DCCM matrices. However, due to the inherent normalization in the DCCM computations, such matrices are not sensitive to the amplitudes of the motions. We therefore complemented the DCCM analysis with covariance and PCA (supplemental Figs. S5 and S6), as well as with differential r.m.s. fluctuations (Fig. 6 and supplemental Fig. S3). The differential r.m.s.
fluctuations refer to the difference between the r.m.s. fluctuation values obtained by superimposing all domains and the domain-specific r.m.s. fluctuations. Although the former r.m.s. fluctuation values reflect both local fluctuations and non-local domain motions, the latter sense mostly local dynamics. Therefore, the differential r.m.s. fluctuations report on the amplitudes of non-local domain motions and are a useful complement to the analysis of interdomain correlations as identified by the DCCM matrices.

Details about the preparation of the initial structures as well as about the MD simulation protocols are provided as supplemental data. The detailed protocols utilized for the validation and the analysis of the MD simulations are also provided as supplemental data.

RESULTS

Dynamics of the Apo/Inactive State of EPAC2

The Closed Topology of the Apo/Inactive State and the Fold of Each Individual Domain Are Stable in the Nanosecond Time Scale—As a first assessment of the simulated MD trajectory starting from the apo/inactive structure, the r.m.s. deviations from both active/inactive initial apo structures (for apo-state simulations) or both active/inactive initial holo structures (for holo-state simulations) are shown, along with r.m.s. deviations of each domain from its respective initial structure. The color coding is shown in each panel. The initial equilibration period of each simulation is indicated as negative times, whereas the production run (used in subsequent analyses) is indicated as positive times. The overlaid initial and final structures from each simulation are shown as insets (black ribbon structure = initial structure; gray ribbon structure = final structure). For the holo/inactive-metastate simulation, the time at which a sudden conformational shift was observed in the NTHB α1 and α2 helices of the CNB domain is also indicated (red dashed line in panel b).
state initial structures (~24 Å), indicating that no active/inactive transition occurs during the time span of the simulation. This interpretation is supported by the observation that the apo/inactive state simulation consistently displayed a ~24 Å r.m.s. deviation from the active state structure (Fig. 2a). It is therefore clear that the MD trajectory remains closer to its initial closed structure than to the open conformation, without a significant change in overall topology. In addition, the fold of each individual domain of the apo/inactive state of EPAC2 does not change significantly during the course of the simulations, as shown by the domain-specific r.m.s. deviations from their respective initial structures (Fig. 2a). The domain-specific r.m.s. deviation values all reached stable maxima of ~3 Å or less early in the initial equilibration period of the simulation (Fig. 2a, negative time), and remained at/near the maximum throughout the rest of the simulation time (Fig. 2a, positive time). Overall, the structures of the individual domains appear to be quite stable. However, the domain-specific r.m.s. deviations do not provide insight into the local dynamics, which are better assessed by backbone N-H order parameters.

Patterns of Local Dynamics in Apo/Inactive EPAC2 and Experimental Validation—The backbone N-H order parameters ($S^2$) for EPAC2 in the apo/inactive state are shown in Fig. 3a and reveal several “hot spots” of local dynamics in each domain. In the CNB domain, the most dynamic regions of the β-core include the PBC, BBR, and β2–3 loop (Fig. 3a), in agreement with previous NMR relaxation studies (18). In addition, an appreciable degree of flexibility was evident in the first α-helix of the CNB domain (NTHB β1; Fig. 3a), again in agreement with the NMR data (18). The extent of the agreement between the NMR data and the MD results is further appreciated by inspecting Fig. 4a, which compares the backbone amide order parameters ($S^2$) measured by NMR for the CNB domain of apo-EPAC1 versus those computed by MD for the corresponding residues of the homologous EPAC2 in the apo/inactive state. Fig. 4a shows that to a large extent the MD simulations capture the qualitative trends of the experimental order parameters. Nine out of 12 local $S^2$ minima are well reproduced by the MD trajectories (Fig. 4a, black arrows). Some deviations between experimental and calculated $S^2$ values are apparent especially at the N and C termini (Fig. 4a), which was expected considering that the NMR data were acquired on a truncated construct of a different EPAC isoform. No NMR data is currently available on the EPAC2 isoform. A particularly notable dynamic hot spot was observed in the region of the NTHB β1 helix that contains two of the three CNB domain residues involved in the ionic latch interface between the CNB and CDC25-HD domains (i.e. residues Gln303 and...
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Asp\textsuperscript{307}; Fig. 1, a and b, and supplemental Fig. S1), suggesting that the ionic latch interface of the apo/inactive state is more dynamic than expected based on the x-ray structure alone (14). To gain further insight into the dynamics of the ionic latch, we examined the time profiles of the ionic latch distances for the apo/inactive state (supplemental Fig. S4). It was found that most of the ionic latch interactions were indeed very dynamic, as reflected by large and highly variable contact distances (supplemental Fig. S4). In particular, the interaction of Gln\textsuperscript{303} with Asp\textsuperscript{883} was highly dynamic, with distances mostly greater than 20 Å (supplemental Fig. S4a), whereas the interactions of Gln\textsuperscript{303} and Asp\textsuperscript{307} with Arg\textsuperscript{886} were noticeably more stable, but still very dynamic (supplemental Fig. S4, a and b). The interactions between Glu\textsuperscript{332} and Arg\textsuperscript{886}, on the other hand, consisted of interactions that were considerably more stable than those among any of the other ionic latch residue pairs (supplemental Fig. S4, c and d).

Another pattern observed in the order parameter (\(S^2\)) profiles shown in Fig. 3a is that regions of lower dynamics were generally clustered in the interior of the protein, whereas regions of higher dynamics resided primarily at the solvent-exposed exterior. Indeed, such a trend is typical for soluble proteins, and as expected, the most dynamic regions were generally within or near loops (Fig. 3a). For instance, the loops composed of residues 465–476 and 725–731 (Fig. 3a, cyan asterisks) displayed particularly low \(S^2\) values, in agreement with the missing electron density for these loops in the x-ray structure (14). In addition, relatively low \(S^2\) values for the RA domain (Fig. 3a and Table 1) indicate that the domains in the catalytic region, in agreement with the poor intradomain packing and high B-factors previously observed for this domain (14). Overall, the dynamic profile predicted by the MD simulations for the apo/inactive state of EPAC is well corroborated by the available experimental data. In addition, MD simulations also provide a glimpse into dynamic features of EPAC that are not experimentally accessible, such as those revealed by the DCCM matrix, which probes both intra- and interdomain correlations.

Intradomain Cross-correlations of Apo/Inactive EPAC2—Upon examination of the DCCM results for the apo/inactive state (Fig. 5a, lower triangle), it was found that the domains in the catalytic region (i.e. REM, RA, and CDC25-HD) all exhibited internal correlations that were primarily positive in value. However, the regulatory CNB domain demonstrated several anticorrelated motions between its \(\alpha\)-helical and \(\beta\)-sheet subdomains, even though the \(\beta\)-sheet subdomain exhibited internal correlations that were entirely positive in value (Fig. 5a, lower triangle).

Interdomain Cross-correlations of Apo/Inactive EPAC2—The examination of interdomain DCCM correlations (Fig. 5a, lower triangle) revealed two key features. First, most of the interdomain correlations observed in the apo/inactive state...
were weak or negative in value (Fig. 5a, lower triangle). For instance, it was unexpectedly found that the CNB domain demonstrated a largely anticorrelated motion relative to the CDC25-HD domain, despite the fact that these two domains are anchored to each other by the IL salt bridges (Fig. 5a, lower triangle). This observation is consistent with the dynamic nature of the ionic latch interactions revealed by the time profiles of the IL distances. Second, the DCCM analysis revealed that the main positive correlations involve residues that either form or are closely associated with the so-called switchboard β-sheet at the boundary between the regulatory and catalytic regions, i.e. CNB domain residues 445–461, REM domain residues 480–500, and residues 910–940 from the CDC25-HD domain helical hairpin (Fig. 5, a, lower triangle, and c). The switchboard site therefore appears to function as a stable interaction hub that anchors the CNB, REM, and CDC25-HD domains to one another (14). Further insight into the interdomain motions is provided by the differential r.m.s. fluctuation analysis (Fig. 6a and supplemental Fig. S3a).

The differential r.m.s. fluctuation profiles (Fig. 6a and supplemental Fig. S3a) indicate that the CNB and RA domains are subject to the largest amplitude motions in the apo/inactive state. We therefore anticipate that the anticorrelated motion between the CNB and RA domains revealed by the DCCM analysis (Fig. 5a, lower triangle) dominates the total motional variance. This result is fully confirmed by the examination of interdomain dynamics through PCA analysis of the covariance matrix (supplemental Fig. S5a). Specifically, the extreme projections of the apo/inactive MD trajectory on the first PCA eigenvector (supplemental Fig. S6a) indicate a “breathing” motion between the CNB and RA domains, involving correlated motions of smaller amplitude in the CDC25-HD domain.

### Dynamics of the Holo/Active State of EPAC2

The Open Topology of the Holo/Active State and the Fold of Each Individual Domain Are Stable in the Nanosecond Time Scale—Analogously to the apo/inactive state, r.m.s. deviations from both active and inactive initial EPAC2 conformations suggested that the holo/active state MD trajectory remains closer to its initial open conformation than to the closed structure, without a significant change in overall topology. This was reflected by considerably larger r.m.s. deviations from the inactive state initial structure (20–25 Å) than from the active state initial structure (4–8 Å) over the course of the simulation (Fig. 6b and c).
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2d), indicating that no active/inactive transition occurs during the 50-ns time span of the simulation. In addition, the structures of the individual domains appeared to be quite stable, as reflected by lower and less variable r.m.s. deviations (≤ 2.5 Å or less) of the individual modules from their respective initial structures during the simulation (Fig. 2d). Notably, the CDC25-HD domain does not deviate significantly from its initial structure despite the absence of the bound Rap1b (Fig. 2d). Overall, the considerable difference between the total and the domain-specific r.m.s. deviation values points to the presence of enhanced interdomain motions in the holo/active state relative to the apo/inactive state (Fig. 2, a and c).

The CNB Domain Exhibits a Markedly Dual Pattern of cAMP-dependent Dynamics in Agreement with the NMR Data, with Enhanced α-Subdomain Dynamics and Quenched β-Subdomain Dynamics in the Holo/Active State Relative to the Apo/Inactive State—Upon examining the backbone order parameters (S^2) computed for the holo/active state, it was found that the α-subdomain, i.e. the NTHB and hinge helix, displayed enhanced dynamics relative to the apo/inactive state (Fig. 3b and Table 1). Particularly notable enhancements were observed within the C-terminal half of the hinge helix and in regions of the NTHB spanning all three residues involved in the ionic latch. In marked contrast to the α-subdomain, the β-core of the CNB displayed quenched dynamics relative to the apo/inactive state, with particularly significant quenching at the PBC, where cAMP docks, and at the adjacent β2–3 loop (Fig. 3b and Table 1). This pattern of α-subdomain enhancement and β-subdomain quenching of dynamics is qualitatively consistent with the trends observed for the cAMP-dependent changes in both the experimental S^2 values and the high-frequency spectral densities J(ω_H + ω_N) measured by 15N NMR for the EPAC1 CNB domain (Fig. 4, b–d) (18), thus further validating the MD simulations. We therefore conclude that MD computations capture the essential features of the cAMP-dependent changes in EPAC dynamics that were experimentally observed for the CNB. In addition, MD provides atomic resolution insight into the dynamic profile of EPAC domains in the catalytic region that are not currently accessible by NMR.

The Catalytic Region Exhibits Multiple Changes in Dynamics in the Holo/Active State Relative to the Apo/Inactive State—In addition to the enhanced dynamics of the CNB α-subdomain, enhancements of dynamics in the holo/active versus apo/inactive state were observed at several sites of the catalytic CDC25-HD domain, as well as parts of the REM and RA domains (Fig. 3b). In general, the changes in local dynamics of the holo/active state in the absence of Rap1b relative to the apo/inactive state span the entire protein, with the largest

FIGURE 6. Ribbon structure map of the differential r.m.s. fluctuation values computed for each state of EPAC2(280–990): (a) apo/inactive, (b) holo/inactive, (c) apo/active, and (d) holo/active. The differential r.m.s. fluctuation values for each state are indicated in the respective structures using a continuous color scale, which ranges from dark blue (r.m.s. fluctuation = 0.0 Å) to red (r.m.s. fluctuation = 3.0 Å). For clarity, the domain boundaries are indicated by purple dashed lines. All structures were generated using PyMOL (Schrödinger, LLC), based on PDB structures 2BYV and 3CF6 from the RCSB Protein Data Bank.
changes generally corresponding to the most dynamic regions of the apo/inactive state (Fig. 3, a and b, and Table 1). For instance, the most notable dynamic enhancements in the CDC25-HD domain occurred in the loop composed of residues 953–961, in agreement with the missing electron density for this loop in the x-ray structure of holo EPAC2 (15), and in the region spanning both CDC25-HD domain residues that in the apo/inactive state were involved in the ionic latch (Fig. 3b). The transition to the holo/active state also causes quenching of dynamics in other regions of all three catalytic region domains. In particular, quenching of dynamics was observed in the lid β-sheet and the helical hairpin loop, which interact to form the switchboard, as well as in the loop composed of residues 725–731, in agreement with the presence of this loop in the active state x-ray structure (Fig. 3b) (15). Furthermore, the RA domain displays the greatest extent of dynamic changes in the catalytic region (Fig. 3b and Table 1), whereas the CDC25-HD domain exhibits the smallest overall extent of perturbation in the catalytic region (Fig. 3b and Table 1). Interestingly, the observed dynamic perturbations include regions forming some of the interactions that selectively stabilize the holo/active versus the apo/inactive state (i.e. the cAMP/CNB, cAMP/REM, and CNB/REM contacts; Fig. 3b, solid vertical black lines). To further explore the dynamics of these holo/active-specific interactions, several distance time profiles were analyzed (supplemental Fig. S7).

In the Holo/Active State Most of the cAMP/CNB Contacts Are Stable, Whereas the CNB/REM Interdomain Interface Is Dynamic—Upon examination of the time profiles for key interactions of cAMP (supplemental Table S2), the hydrogen bonds of cAMP with residues of the PBC (i.e. Gly404, Ala407, Arg414, and Ala415) and with Lys450 of the lid β-sheet proved to be quite stable, as suggested by contact distances persistently residing at/near values compatible with formation of hydrogen bonds and/or near the distance values observed in the x-ray structure. In addition, the hydrophobic contacts of cAMP with the PBC (Ala415 and Ala416), lid (Leu449), Phe467, and the BBR residue Val386 were also quite stable, as indicated by distances persistently residing at/near van der Waals contact range and/or near the distance values in the x-ray structure (supplemental Table S3). The only contacts between CAMP and the CNB that exhibited some degree of dynamics were those involving residues Ile388 and Val394 of the BBR (supplemental Fig. S7a). In particular, Ile388 showed a clear drift in average cAMP contact distance over the course of the simulation, with overall variation over a range of ~4 Å (supplemental Fig. S7a). The Val394 side chain demonstrated two sudden flips in orientation, between which one of the side chain terminal methyl groups (γ1 and γ2 methyls) formed a very stable contact with cAMP (supplemental Fig. S7a).

Contrary to the majority of the cAMP/CNB contacts, the CNB/REM interface appeared quite dynamic. The PBC lid hydrogen bonds established by Lys489 of the lid α-helix (α1 of the REM domain) was found to be dynamic, as indicated by a highly variable contact distance (supplemental Fig. S7b). Overall, the time-dependent distance profiles of the holo/active state of EPAC suggest that the widespread dynamic changes of the catalytic region affect also its interface with the regulatory CNB domain.

The Dynamics of the Holo/Active State Are Extensively Correlated—Another unique feature of the holo/active state is a markedly distinct DCCM matrix in terms of both patterns and magnitudes of positive and negative correlations, which are clearly different from any other state of the protein, as illustrated in Fig. 5b (upper half). For instance, marked changes in the correlation magnitudes and patterns are clearly apparent when comparing the DCCM of the holo/active state with the apo/inactive state (Fig. 5, b, upper half, versus a, lower half). First, more extensive positive correlations were observed within the REM domain and the CNB domain demonstrated mostly positive internal correlations in the holo/active state (Fig. 5b, upper half), without the extensive negative correlations between the two subdomains observed for the apo/inactive state (Fig. 5a, lower half). In addition, mostly positive correlations were also observed between the CNB and REM domains, although they were visibly weaker than the intradomain correlations (Fig. 5b, upper half). Furthermore, whereas the CDC25-HD domain demonstrated several markedly negative correlations with the other domains, some well defined positive correlations were also visible and appeared to correspond to regions of the CDC25-HD domain forming contacts with the other domains (Fig. 5b, upper half). For instance, positive correlations observed with the CNB and REM domains corresponded to the helical hairpin, as well as other CDC25-HD domain elements interacting with the helical hairpin (Fig. 5, b, upper half, and d). Finally, it is notable that the RA domain is the EPAC2 module with the least positive interdomain correlations (Fig. 5b, upper half), with the few positive correlations involving the RA domain confined to the RA/CDC25-HD contacts (Fig. 5, b, upper half, and d).

The overall high degree of correlation in the fluctuations of the holo/active state is also confirmed by PCA analysis of the covariance matrices (supplemental Fig. S5d). Specifically, when the MD trajectory was projected onto the space spanned by the first six eigenvectors, with all residues superimposed except for the most flexible regions (i.e. the first 20 amino acids of the NTHB α1 helix and the mostly unstructured loop connecting the CNB and REM domains), the large anticorrelated oscillation that in the apo/inactive state was observed for the CNB and RA domains is now reduced in amplitude, but correlated motions are apparent throughout the structure, particularly in the catalytic region (supplemental Fig. S6b). Furthermore, the presence of collective motions involving the entire catalytic region is also independently confirmed by the differential r.m.s. fluctuation profiles (Fig. 6d and supplemental Fig. S3b), which indicate that the REM, RA, and CDC25-HD modules are subject to domain motions with significantly increased amplitude in the holo/active state relative to the apo/inactive state (Fig. 6a and supplemental Fig. S3a).
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Dynamics of the Rap1b-bound Holo/Active State of EPAC2

The Open Topology of the Rap1b-bound Holo/Active State and the Fold of Each Individual Domain Are Stable in the Nanosecond Time Scale—As with the holo/active state, the r.m.s. deviations from both active and inactive initial EPAC2 conformations suggest that the Rap1b-bound holo/active state remains closer to its initial open structure than to the closed structure, without a significant change in overall EPAC2 topology (supplemental Fig. S8a). In addition, the structures of the individual domains appeared to be quite stable, as reflected by low (i.e. < ~3 Å) r.m.s. deviations during the simulation (supplemental Fig. S8a, positive time).

The Binding of Rap1b Substrate to the Holo/Active State Causes a Partial Reversal of Changes in Dynamics from the Apo/Inactive State—Upon examining backbone order parameters (\(S^2\)) computed for the holo/active state with bound Rap1b (Fig. 3e and Table 1), it was found that the addition of Rap1b to the holo/active state resulted in a quenching of dynamics not only for most of the CDC25-HD domain, which contacts Rap1b directly, but also for the distal CNB domain where dynamics in both the \(\alpha\)- and \(\beta\)-subdomains are quenched upon Rap1b binding to the CDC25-HD. Unlike the CDC25-HD and CNB domains, an enhancement of dynamics was observed throughout most of the RA domain (Fig. 3e and Table 1). Overall, it appears therefore that Rap1b causes a partial reversal of the dynamic changes observed in the apo/inactive to holo/active transition. This trend is also confirmed at the level of the DCCM and differential r.m.s. fluctuation analyses.

Upon examination of the DCCM results for the holo/active state with bound Rap1b (supplemental Fig. S8b), it was found that the strong correlations and anticorrelations observed within the holo/active state (Fig. 5b, upper half) became weakened upon Rap1b binding. In fact, the Rap1b-bound holo/active state demonstrated DCCM magnitudes that were more comparable with those of the apo/inactive state (Fig. 5 and supplemental Fig. S8b). In addition, the differential r.m.s. fluctuation analysis (supplemental Fig. S8c) indicated that the amplitudes of the non-local motions for the REM and CDC25-HD domains are reduced upon substrate binding to values more similar to those observed for the apo/inactive state (Fig. 6a).

Changes in Dynamics Observed for the Holo/Inactive Metastate of EPAC2

The Closed Topology of the Holo/Inactive Metastate and the Fold of Each Individual Domain Are Quite Stable in the Nanosecond Time Scale—As with the apo/inactive state, the r.m.s. deviations from both active and inactive initial EPAC2 conformations suggested that, despite the presence of cAMP, the holo/inactive metastate MD trajectory remains closer to its initial closed structure than to the open structure, without a significant change in overall topology in the nanosecond time scale (Fig. 2b). In addition, the structures of the individual domains appeared to be quite stable with the exception of the CNB domain, which demonstrated a notable sudden increase of 1 Å at ~37 ns into the 50-ns production run period (Fig. 2b).

Visualization of structures before and after this time point indicated a shift in the position of NTHB helices \(\alpha1\) and \(\alpha2\) relative to the rest of the CNB domain, explaining the observed r.m.s. deviation increase (Fig. 2b, inset). Overall, these observations suggest that transition from the holo/inactive to the holo/active state occurs in a time scale longer than tens of nanoseconds, which is beyond the scope of the MD simulations analyzed here. However, the absence of interstate transitions during the MD trajectory provides the opportunity of capturing the distinct nanosecond/subnanosecond dynamic profile of the holo/inactive metastate, which is otherwise challenging to trap and characterize experimentally.

The CNB and RA Dynamics Are Perturbed in the Holo/Inactive Metastate Relative to the Apo/Inactive State, with Lesser Overall Changes in the Other Domains—Upon examining the backbone order parameters (\(S^2\)), it was found that relative to the apo/inactive state, the holo/inactive metastate demonstrated a quenching of dynamics within the \(\beta\)-core, with particularly significant quenching at the PBC, as expected, and at the adjacent \(\beta2–3\) loop (Fig. 3d and Table 1). Meanwhile, dynamic enhancements occurred throughout most of the NTHB, including the regions spanning all three CNB domain residues involved in the ionic latch (Fig. 3d and Table 1). Similarly to the CNB \(\alpha\)-subdomain, the RA domain exhibited primarily enhanced local dynamics (Fig. 3d and Table 1), while a quenching of dynamics was observed in the lid \(\beta\)-sheet and parts of the CDC25-HD domain, including a partial quench of the helical hairpin loop that forms part of the switchboard (Fig. 3d). Overall, the holo/active state displays the lowest average order parameters (\(S^2\)) among the five states investigated here (Table 1), suggesting that the holo/inactive state benefits from an entropic stabilization. Further insight into the dynamics in the holo/inactive state is provided by the comparative analysis of the DCCMs of Fig. 5a.

The Holo/Inactive Metastate Promotes a De-correlation of the CNB/CDC25-HD Dynamics—Upon examination of the DCCM results for the apo/inactive and holo/inactive states (Fig. 5a), it was found that the anticorrelations between the NTHB and CDC25-HD domain almost vanished and the anticorrelations between the \(\beta\)-core and CDC25-HD domain observed for the apo/inactive state became somewhat diminished in the holo/inactive metastate (Fig. 5a, upper half). In addition, the positive correlations between the NTHB and \(\beta\)-core of the CNB domain became diminished in the holo/inactive metastate (Fig. 5a, upper half). However, these observations point to a loosening effect on the IL interface formed by the NTHB upon transition from the apo/inactive state to the holo/inactive metastate. This result is also consistent with the increased \(\alpha\)-subdomain local dynamics in the holo/inactive versus apo/inactive state (Table 1) and with the aforementioned shift in the positions of the NTHB \(\alpha1\) and \(\alpha2\) helices relative to the CNB \(\beta\)-core, as revealed by the CNB-specific r.m.s. deviation time profile of the holo/inactive simulation (Fig. 2b). Furthermore, the apo/inactive to holo/inactive transition results in enhanced non-local dynamics for the RA domain and CNB domain \(\beta\)-core, as indicated by the differential r.m.s. fluctuation analysis (Fig. 6b and supplemental Fig. S3d).
Changes in Dynamics Observed for the Apo/Active Metastate of EPAC2

The Open Topology of the Apo/Active Metastate and the Fold of Each Individual Domain Are Stable in the Nanosecond Time Scale—As with the holo/active state, the r.m.s. deviations from both active and inactive initial EPAC2 conformations suggested that the apo/active metastate MD trajectory remains closer to its initial open structure than to the closed structure, without a significant change in overall topology despite the absence of cAMP (Fig. 2c). In addition, the structures of the individual domains appeared to be quite stable, as reflected by lower r.m.s. deviations during the simulation (Fig. 2c, positive time). Again, the absence of interstate transitions during the course of the MD trajectory provides the opportunity to capture the dynamic features of the otherwise elusive apo/active metastate.

The Apo/Active Metastate Demonstrates Mostly Quenched Dynamics, Relative to the Apo/Inactive State—Upon examining the backbone order parameters (S^2), it was found that relative to the apo/inactive state, the apo/active metastate demonstrated a quenching of dynamics in the β-core and a partial enhancement of dynamics confined to most of the α-subdomain, i.e., the NTHB and hinge helix (Fig. 3c and Table 1). Meanwhile, a quenching of dynamics was observed in the RA domain, as well as in the lid β-sheet and helical hairpin loop (Fig. 3c and Table 1). Overall, the apo/active state displays the highest average order parameters (S^2) among the states investigated here (Table 1), suggesting that the apo/active state is associated with an entropic penalty.

Correlations Among Residue Movements Are Less Extensive in the Apo/Active Metastate Than in the Holo/Active State—Upon examination of the DCCM results, it was found that unlike the holo/active state, the apo/active metastate demonstrated correlations somewhat comparable in magnitude to those observed for the two overall weakly correlated inactive states (Fig. 5a and b). Specifically, the correlation of CNB and lid/REM domain movements observed in the holo/active state was abolished in the apo/active metastate, suggesting a de-correlation across the CNB/REM domain interface upon cAMP dissociation (Fig. 5b). Furthermore, the large amplitude domain motions observed for the CNB and CR in the holo/active states are also to a large extent lost in the apo/active state (Fig. 6, c and d, and supplemental Fig. S3, b and c), thus confirming the loss of interdomain fluctuations upon cAMP dissociation and further supporting the existence of an entropic penalty associated with the apo/active metastate.

DISCUSSION

The MD Simulations Are Validated by the Available Experimental Data and Also Provide Novel Insight into Functional Dynamic Features That Have Remained Elusive to Experimental Methods—The first notable feature of the MD simulations presented here is that they capture the key trends in the dynamic profiles experimentally probed by NMR for the CNB domain in both the apo and holo states. Such agreement has two key implications. First, it suggests that the NMR data previously obtained for a single domain construct (18) are relevant also in the context of full-length EPAC. Second, the ability of MD simulations to reproduce critical experimental trends validates the reliability of MD trajectories in qualitatively capturing the EPAC dynamic profile and its cAMP dependence. The MD simulations thus provide an unprecedented view of EPAC dynamics, which is particularly valuable for the motions that have remained so far challenging to probe experimentally at residue resolution, such as those in the catalytic region and in the apo/active and holo/inactive metastates (Fig. 1b). The major results obtained from the five simulations of EPAC2 (280–990) (supplemental Table S1) are summarized schematically in Fig. 7 and supplemental Fig. S8d. The dynamic fluctuations of EPAC summarized in Fig. 7 and supplemental Fig. S8d have several pivotal implications for both the autoinhibition and the cAMP-dependent activation of EPAC. We will first address the role of dynamics in the latter.

Dynamics in the cAMP-dependent Activation of EPAC

EPAC Activation Involves a Reciprocal Long-range RR/CR Dynamic Cross-talk, Assisted by Highly Correlated Motions in the Switchboard Region and Relevant for Substrate Binding—Upon conversion from the apo/inactive state to the holo/active state, several changes in local dynamics occur throughout EPAC (Fig. 7), indicating that the cAMP-dependent changes in dynamics that were previously observed for the CNB domain (18) are actually not confined solely to the RR, but extend to the CR as well (Fig. 7). Specifically, for both the RA domain and the helical hairpin (HP) in the CR, the local dynamics are quenched in the holo/active state relative to the apo/inactive state (Fig. 7). The reduction of HP dynamics explains the reduced solvent accessibility observed for this region upon cAMP binding in recent H/D exchange MS experiments (39). Furthermore, the cAMP-dependent dynamic quenching in the HP region reduces the entropic penalty for the binding of the Rap1b substate to the HP (15), which is likely to promote the Rap1b/CDC25-HD interaction (40).

The perturbations in the CR caused by cAMP binding to the RR are not limited only to local dynamics, but they also affect the collective interdomain dynamics. For instance, Figs. 6 and 7 show that cAMP binding amplifies the amplitude of the non-local RA and CDC25-HD domain dynamics. Remarkably, such long range cAMP-dependent effects on non-local CR dynamics occur irrespective of whether cAMP binds to the inactive or active conformations of EPAC (Figs. 6 and 7), suggesting that cAMP controls the function of the catalytic region of EPAC not only by stabilizing the open active structure as previously thought (14–17), but also by contributing directly to the modulation of CR dynamics.

The MD simulations also indicate that the cross-talk between the RR and CR of EPAC is reciprocal. Not only does cAMP binding to the RR modulate CR dynamics, but Rap1b binding to the CR also modulates the RR dynamics (Fig. 3e, supplemental Fig. S8, c and d, and Table 1). These long-range effects of Rap1b binding are fully consistent with the highly correlated character of the dynamics in the holo/active state (Fig. 5b, upper half), which serves the purpose of promoting allosteric signal propagation across the different EPAC domains. In particular, the switchboard region emerges as a
stable hub of interactions that anchor together three critical EPAC domains (the CNB, REM, and catalytic CDC25-HD domains) and remain highly correlated in all EPAC states simulated here (Fig. 5), mediating the dynamical cross-talk between the regulatory and catalytic regions of EPAC. Additional insight into the mechanism of EPAC activation was obtained through a further analysis of the dynamic profiles of the two metastates (i.e., holo/inactive and apo/active), which are critical to dissect the relative contributions of cAMP binding and closed to open conformational transitions to the cAMP-dependent EPAC activation.

**EPAC Activation Involves Dynamic Contributions from Both cAMP Binding and Transition to the Active Conformation**—The dynamic profiles of the holo/active and apo/active metastates (Figs. 3 and 7, and Table 1) suggest that both cAMP binding and transition of EPAC to the active conformation contribute to the changes in local dynamics occurring upon EPAC activation. However, the magnitudes of the distinct contributions of the two metastates appear to be domain specific. For instance, in the case of the CNB domain, the pattern of α-subdomain enhancement and β-core quenching in local dynamics reported for the apo/active to holo/active transition is also observed in both metastates (Figs. 3 and 7, and Table 1), whereas in the case of the CR domains, the quenching in local dynamics associated with the apo/active to holo/active transition (Table 1) appears to arise predominantly from the apo/active metastate (Table 1). Such insight provided by the MD simulations into the metastate dynamics has important implications for the significance of the induced fit versus conformational selection pathways of allostery, which proceed through the holo/inactive and apo/active intermediates, respectively (Fig. 1b) (26, 29–33).
cAMP. The conformational selection pathway is a plausible model of EPAC alloster because the apo/active metastate has a higher binding affinity for cAMP than the apo/inactive state, due to better geometric complementarity (29–31) and reduced binding site dynamics (Fig. 7). However, it should be considered that the r.m.s. deviation, order parameter ($S^2$), and DCCM results consistently point to a decoupling of dynamics across the ionic latch interface in the holo/inactive metastate, i.e. the binding of cAMP to the inactive state triggers a destabilization of the ionic latch interface between the CNB and CDC25-HD domains. This means that under conditions in which the induced fit pathway prevails, i.e. when cAMP binding precedes the conformational transition from inactive to active structures, cAMP primes the EPAC system for the closed to open transition by loosening the ionic latch interface prior to the actual conversion from inactive to active conformations (Fig. 7). Furthermore, the holo/inactive metastate, unlike the apo/active metastate, is entropically stabilized by local dynamics as indicated by a minimal average $S^2$ value (Table 1). We therefore conclude that although the conformational selection pathway can account for alloster in EPAC, the induced fit mechanism should not be a priori ruled out either. Indeed, it has been shown that both pathways are viable depending on the experimental conditions (29, 32, 33), and previous kinetic and NMR studies have suggested that ligand-associated conformational changes occur significantly through both pathways under a range of experimental conditions (41, 42). A possible hybrid mechanism for EPAC alloster should then be considered and is discussed below.

**A Hybrid Induced Fit/Conformational Selection Mechanism for EPAC Alloster Provides a Low Free-energy Activation Pathway**—The finding that in the holo/inactive state the IL interface is weakened by cAMP binding due to an increase in its entropic penalty suggests a possible low free-energy pathway for the apo/inactive to holo/active transition. Specifically, low affinity binding of cAMP to the apo/inactive state leads to the transient formation of a holo/inactive intermediate, which may serve as a transitory complex in which the IL is weakened and primed for transition to the active conformation (Fig. 7). Due to the low binding affinity of cAMP for the inactive conformation of EPAC, it cannot be ruled out that during the conformational transition CAMP may dissociate from EPAC, leading to the formation of the apo/active intermediate (Fig. 7). However, this metastate can in turn re-associate with CAMP with significantly higher affinity, eventually resulting in the holo/active state (Fig. 7).

A remarkable feature of the proposed hybrid activation pathway is that in each successive step unfavorable entropic losses are compensated, at least in part, by favorable entropic gains. For instance, in the apo/inactive to holo/inactive transition a partial quenching of local $b$-core and switchboard dynamics is compensated by an enhancement in the local dynamics of the NTHB and the RA domain (Fig. 7, a and b). In the holo/inactive to apo/active step, entropic losses in the $b$-core, CDC25-HD, and RA domains are partially offset by entropic gains linked to the release of CAMP and to the increased dynamics of the CNB $\alpha$-subdomain (Fig. 7, b and c). Last but not least, in the apo/active to holo/active transition the entropic penalty due to the loss of free CAMP and to the quenching of REM domain dynamics is at least partially compensated by enhancements in the dynamics of the CDC25-HD and RA domains (Fig. 7, c and d). It is notable that such entropic compensations not only apply to CAMP binding/release and local protein dynamics, but extend to protein interdomain motions as well (Figs. 6 and 7). We therefore propose that such stepwise compensatory changes in protein dynamics serve the purpose of reducing excessive entropic losses along the apo/inactive to holo/active conversion pathway, thereby facilitating the minimization of free energy during the CAMP-dependent activation (Fig. 7). Of course, a similar consideration would apply for the reverse transitions that occur during the holo/active to apo/inactive conversion and therefore the hybrid pathway proposed above may also promote the reversibility of EPAC activation in response to CAMP. Such reversibility is critical for the timely termination of the signal relayed by CAMP in the EPAC-dependent signaling pathways.

**Dynamics in the Autoinhibition of EPAC**

Another notable conclusion emerging from the MD simulations presented here is that the two key sets of autoinhibitory interactions that selectively stabilize the apo/inactive state, i.e. the hydrophobic hinge and the ionic latch, are both significantly affected by dynamics. The mechanism of EPAC autoinhibition should thus be revisited in light of the dynamic profiles revealed by the MD trajectories.

**A Dynamic Hydrophobic Hinge**—The high degree of dynamics predicted for the PBC in the apo/inactive state calls for a re-examination of the “hydrophobic hinge” hypothesis previously proposed to rationalize the stabilization of the closed EPAC conformation. The original hypothesis posited that in the inactive (closed) topology PBC residue Leu$^{408}$ and the hinge helix residue Phe$^{435}$ are oriented such that they create inhibitory steric hindrance between the PBC and hinge helix. As a result, the rotation of the hinge helix toward the PBC that occurs during conversion to the active (open) conformation is blocked in the absence of CAMP, inhibiting the conformational shift (14, 15). However, this static view of the Leu$^{408}$/Phe$^{435}$ interaction does not take into account the highly dynamic nature of the PBC in the apo/inactive state (Fig. 3a). An additional explanation reconciling the pivotal inhibitory role of the conserved Leu$^{408}$/Phe$^{435}$ residues with the high degree of dynamics of the PBC is that the tight Leu$^{408}$/Phe$^{435}$ packing is unfavorable in the absence of CAMP as it would result in a significant entropic loss for the PBC. This interpretation is supported by the observation that the dynamics within the PBC, as well as other key $b$-core sites, were found to be quenched not only by CAMP binding, but also by the transition of EPAC to its active conformation (Figs. 3 and 7, a and c, and Table 1). Remarkably, the apo/active state displays the highest average $S^2$ value for the $b$-core as well as for the full-length construct (Table 1), further confirming that the transition from inactive to active conformations in the absence of CAMP is associated with a significant penalty in terms of decreased conformational entropy.
Intrinsic Dynamics of EPAC

Dynamics Sensitize the IL to cAMP by Causing a Partial Weakening of Selected Functional IL Salt Bridges—The MD simulation of the apo/inactive state suggests that at least part of the IL interface between the CNB and CDC25-HD domains is quite labile. The order parameter (S^2) in the IL region and the time profiles of the IL interaction distances consistently point to the IL interface being overall quite dynamic in the apo/inactive state, with the IL interactions mediated by Gln^{303} and Asp^{307} being considerably less stable than the Gln^{332}/Arg^{886} IL salt bridge (Fig. 3a and supplemental Fig. S4). This pattern is likely due in part to the presence of a Gly-Pro residue including the flanking residues. However, it should be noted that despite their flexibility, the IL residues Gln^{303} and Asp^{307} still play a role in the ionic latch interface, because when the transient interactions formed by these residues are perturbed through the Δ306 deletion mutation, the maximal activity of EPAC2 is enhanced 5-fold (14). Thus, the apparent lability of the contacts mediated by Gln^{303} and Asp^{307} may serve the purpose of sensitizing the IL RR/CR inhibitory interface of EPAC to dynamic perturbations arising from cAMP binding, while at the same time avoiding the complete severance of the IL interface, thereby promoting efficient yet cAMP-dependent EPAC activation (43, 44). This interpretation is also consistent with the anticorrelated fluctuations of the CNB and CDC25-HD domains (Fig. 5a, lower triangle), which suggest the presence of breathing motions between the CNB domain and the catalytic region. Such motions may assist the initial phase of the opening of the CNB/CDC25-HD interface that occurs during the cAMP-dependent transition to the active conformation.

Concluding Remarks—We have performed five 50-ns MD simulations in explicit solvent on a ~700-residue functionally integral construct of EPAC2, starting from the key states of the thermodynamic cycle that describes the coupled binding/activation equilibria of EPAC. The MD results are not only consistent with the currently available experimental data on the dynamics of EPAC, but they also provide an unprecedented insight on picosecond–nanosecond dynamic attributes that have so far remained largely elusive to experimental approaches. Such dynamic features provide the basis for a molecular model explaining several key aspects of EPAC activation and autoinhibition. Specifically, it was found that cAMP binding causes an extensive long-range perturbation of dynamics in the distal catalytic region, which assists the Rap1b substrate recognition. In addition, analysis of the apo/active and holo/inactive cross-states (”metastates”) suggested a possible hybrid mechanism of EPAC allostery that incorporates elements of both induced fit and conformational selection mechanisms, resulting in a low free-energy pathway by which EPAC is effectively and reversibly activated through an entropy compensation strategy. Finally, the simulations revealed that the autoinhibitory interactions stabilizing the apo/inactive state of EPAC are significantly more dynamic than previously anticipated, suggesting that dynamics play a key role in controlling the stability of the apo/inactive state. Such dynamic fine-tuning is critical for the optimal sensitization of the autoinhibitory interactions to the cAMP allostERIC effector, because it ensures that the autoinhibitory interactions are stable enough to avoid constitutive activation in the absence of cAMP, whereas at the same time preventing an excessive stabilization that would compromise an effective cAMP-dependent activation of EPAC. It is anticipated that the models proposed here are of general applicability to multidomain signaling proteins controlled by allosteric effectors.

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