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Molecular Simulations Reveal an Unresolved Conformation of the Type IA Protein Kinase A Regulatory Subunit and Suggest Its Role in the cAMP Regulatory Mechanism

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Supporting Information

ABSTRACT: We identify a previously unresolved, unrecognized, and highly stable conformation of the protein kinase A (PKA) regulatory subunit RIα. This conformation, which we term the "Flipback" structure, bridges conflicting characteristics in crystallographic structures and solution experiments of the PKA RIα heterotetramer. Our simulations reveal a hinge residue, G235, in the B/C helix that is conserved through all isoforms of RI. Brownian dynamics simulations suggest that the Flipback conformation plays a role in cAMP association to the A domain of the R subunit.

Protein kinase A (PKA) is a ubiquitous eukaryotic kinase that modulates the function of proteins through targeted phosphorylation. An ancient cellular second messenger, cAMP, modulates the activity of PKA in a diverse set of biological processes, from synaptic plasticity[1] to cardiac signaling.[2] Inactive PKA exists as a heterotetramer (R_2C_2), where two catalytic (C) subunits are maintained in the inactive state by binding to the regulatory (R) subunit homodimer.[3] In response to extracellular signals like adrenaline,[4] adenylyl cyclase activity leads to an increase in the level of cAMP. When four molecules of cAMP bind to the cyclic nucleotide binding domains (CBD) of the R dimer, a global conformational change in R occurs, releasing two active C subunits, free to phosphorylate protein targets. This activation cycle is the generalized mechanism for all nonredundant forms of R (RIα, RIβ, RIIα, and RIIβ) and C (Cα, Cβ, and Cγ). PKA subtypes are expressed in every cell and encoded by separate genes, differing in structure, activity, and cellular localization.[5,6] Thus, elucidating the structural organization of PKA complexes and their relationship to activation by cAMP is important for the development of novel therapeutics and the understanding of PKA's fundamental biochemistry.

Structural biologists have elucidated PKA isoforms in various stages of the activation cycle. Although the heterotetrameric (R_2C_2) structure of the type IA protein kinase, RIα, has never been fully resolved, it has been the subject of several studies and models. Su et al. crystallized RIα in complex with two molecules of cAMP.[7] This structure of the PKA R subunit at the end of the activation cycle, known as the "Bound" conformation or "B form" (R^{Bound}), revealed the amino acid residues that are important for coordinating cAMP. Each PKA R subunit has two cyclic nucleotide binding domains (CBD-A and CBD-B) joined by a helical moiety known as the B/C helix. The CBD is a conserved sensor of cAMP and composed of noncontiguous α helices and β barrel subdomains. At the N-terminus, a 3_{10} helix–loop region (N3A motif[8]) is followed by a β sandwich containing cAMP binding residues, and a terminal helical region (B and C-terminal helices). The cAMP-bound structure has served as an invaluable resource for understanding cAMP activation through molecular simulations[9] and experiments.

The first structure of the RC "holoenzyme" heterodimer featured the C subunit in complex with RIα CBD-A (R_AC).[10] Point mutations made at a key cAMP-interacting residue, $Arg333^R$ in CBD-B, led to the crystallization of the dual-domain R subunit with the C subunit,[11] RC^{Holo}. The R subunit "Holoenzyme" conformation or "H form", R^{Holo}, is different from the cAMP-bound structure, "Bound" or "B form", R^{Bound} (Figure 1). The Bound conformation is globular, with CBD contacts resulting from a bent B/C/C′ helix. In B form, the B/C helix breaks at L233 and Y244 (Figure 1A), bringing CBD-A into contact with $N3A^B$. In the RC^{Holo} structure, the R subunit wraps around the C subunit (Figure 1B), and the CBDs are separated by an extended B/C helix. In the absence of the C subunit, the H form is stabilized by cAMP analogues.[12] The cAMP-bound, "B" conformation cannot physically accommodate the C subunit because $N3A^B$ interacts with C in RC^{Holo} while $N3A^B$ interacts with CBD-A in R^{Bound} (Figure 1).

Despite extensive efforts, the structure of the full-length (R_{AB}), wild-type type IA (RIα) PKA heterotetramer remains elusive. Structural models of the tetramer are available[13] but not fully consistent with the structure and dynamics of PKA in solution. Specifically, the heterodimeric mutant R333K crystal structure has an R/C interface larger than described by hydrogen/deuterium exchange mass spectrometry (HDXMS) (Figure 1B).[14−16]

Here, we use molecular simulation techniques to make sense of discordant experimental findings from X-ray crystallography, scattering, and HDXMS experiments. We present a novel conformation of the regulatory subunit that resolves these disparities, the "Flipback" or "F form". Finally, we use electrostatic descriptions of the biomolecules to understand the effects of structural changes in cAMP association; offering a role for Flipback in the regulatory mechanism of PKA RIα.

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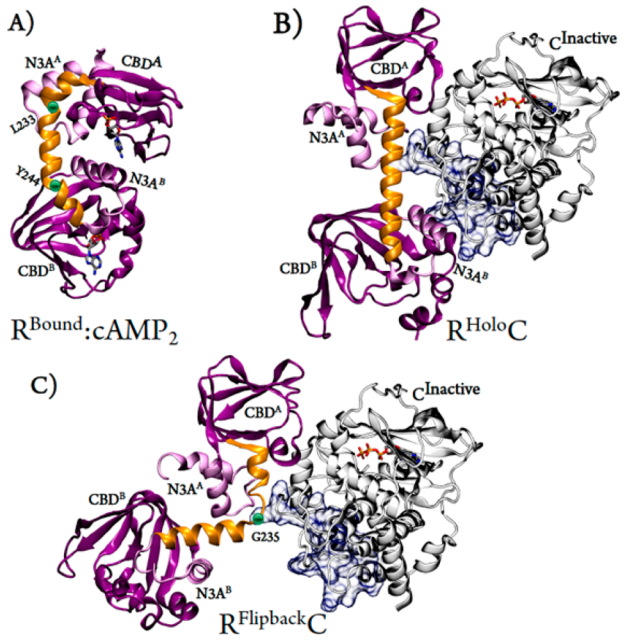


Figure 1. Comparison of the novel Flipback heterodimer with resolved PKARIα conformations. (A) (PDB ID 1RGS) R^{Bound}:$cAMP_2$, the Bound or B conformation, RIα (purple ribbon) with two molecules of cAMP (licorice). (B) (PDB ID 2QCS) R^{Holo}:C, the Holo or H form, RIα (purple ribbon) in complex with the C subunit (white ribbon). (C) $R^{Flipback}$:C, the Flipback conformation or F form, an MD-derived metastable state, aligned with the Cα subunit (white ribbon). B/C helices are shown as gold ribbons. The N3A motifs of A and B ($N3A^A$ and $N3A^B$) are colored light purple. In white, the C subunit is shown with ATP (licorice). A blue surface representation highlights the H/D exchanging regions of the C subunit measured by HDXMS.

To understand the flexibility of the apo WT R subunit, we performed all-atom molecular dynamics (MD) simulations starting from the H conformation in the absence of the C subunit and cAMP. We simulated WT H in five 200 ns replicates using the AMBER[17] force field in the *NTP* ensemble at 310 K. MD of WT-R^{Holo} reveals a very flexible B/C helix as observed in other simulations.[18,19] Mutagenesis of B/C helix residues recently showed pronounced effects on PKA activation.[14]

Our MD simulations reveal a unique, stable conformation of the R subunit (Figure 1C and Figure S1), which we call the "Flipback" conformation or "F form", $R^{Flipback}$. Like R^{Bound}, $R^{Flipback}$ features interdomain (CBD-A/B) interactions and a break in the B/C helix. However, $R^{Flipback}$ breaks in the opposite direction, with interactions between alternate CBDs and N3A motifs. R^B uses contacts between $N3A^B$ and CBD-A, while R^F uses $N3A^A$ to interact with CBD-B (Figure 1B,C). In $R^{Flipback}$, the B/C helix breaks at $Gly235^R$ early in the trajectory (~20 ns), bringing the CBDs in contact for the rest of the simulation in a stable conformation (Figure S1). Mutations limiting the flexibility of the B/C helix (G235P) result in poor C subunit binding,[20] promoting activation. Residues 230–238 of the B/C helix exhibit nearly equal hydrogen bond propensities in the holoenzyme, cAMP-bound, and cAMP-free forms,[15] suggesting the B/C helix is equally flexible in all structures. Gly235 is conserved among all forms of RI.[21] Thus, a helical break at this position may be important in the activation of other type I PKA R subunits.

When $R^{Flipback}$ is aligned with CBD-A of RC^{Holo}, it is apparent that the Flipback conformation can accommodate the C subunit, unlike R^{Bound}. We created a PKA R_2C_2 model using the F form ($R_2C_2^{Flipback}$). As F and H are the only known conformations that can accommodate the C subunit, we were curious to understand how conformational changes in R affect cAMP association. The diffusion of cAMP, a polar molecule, is likely influenced by long-range electrostatic forces that are estimated computationally by Brownian dynamics (BD) simulation methods.[22−24]

Using the existing[13] and newly constructed models of the PKA heterotetramers and heterodimers ($R_2C_2^{Holo}$, $R_2C_2^{Flipback}$, RC^{Holo}, and $RC^{Flipback}$, respectively), we examine the relative rate of cAMP encounter to individual CBDs via BD simulations to determine association rates ($k_{association}$). An "encounter complex" is formed when a specified distance between a set of atoms is reached. We chose three conserved residues to define encounter complexes in CBD-A/B: Val184/300, Glu200/324, and Arg209/333 (Figure S2 and Table S1). Using BrownDye,[25] we compare the effect of Holo and Flipback on cAMP association to tetrameric and heterodimeric conformations.

Predicted BD rates are remarkably consistent with apparent k_{on} values from experiments: 4.52×10^6 and 1.00×10^5 (CBD-B and CBD-A, respectively).[26] The fastest rate of cAMP encounter with PKA tetramers is that for $R_2C_2^{Holo}$ CBD-B (~10^7 M^{-1} s^{-1}), while CBD-A association is slowest [~10^5 M^{-1} s^{-1} (see Table 1)]. Domain B preference in H validates the

Table 1. Rates of Association of cAMP with Cyclic Nucleotide Binding Domains of PKA Complexes

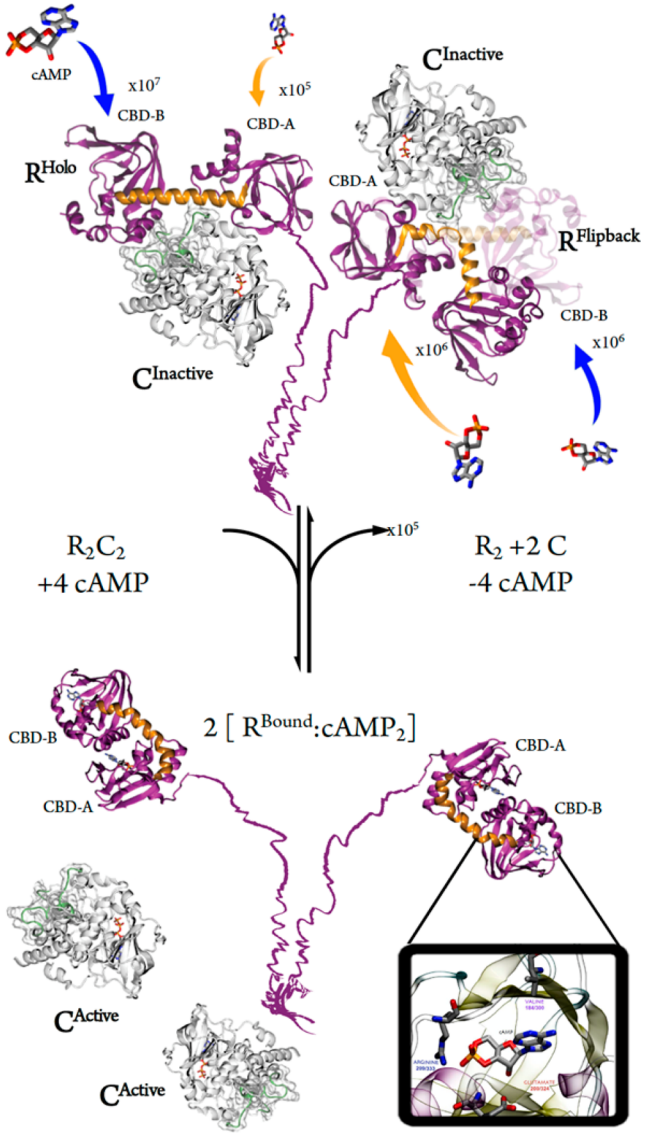
cAMP binding domain (CBD)	PKA conformation	$k_{association}$ (M^{-1} s^{-1})
A	$R_2C_2^{Holo}$	3.07×10^5
B	$R_2C_2^{Holo}$	2.57×10^7
A	$R_2C_2^{Flipback}$	4.29×10^6
B	$R_2C_2^{Flipback}$	4.16×10^6
A	RC^{Holo}	2.50×10^4
B	RC^{Holo}	2.72×10^4
A	$RC^{Flipback}$	1.38×10^8
B	$RC^{Flipback}$	1.98×10^6

standing "gatekeeper" theory of PKA RIα, which holds that cAMP binds to CBD-B first.[27] Domain B preference is neutralized in $R_2C_2^{Flipback}$, where both CBD-A and CBD-B bind on the order of ~10^6 M^{-1} s^{-1} (Table 1 and Scheme 1).

Electrostatically, the systems differ in the distribution of charge on the surfaces of PKA complexes. Flipback has a more electropositive CBD-A than Holo (Figure S4), yielding a higher cAMP association rate. The phenomenological preference for CBD-A in Flipback is most pronounced in heterodimers, with association rates being 2−4 orders of magnitude higher in F. This difference in association rates is due to the very different electrostatic potential surfaces of the heterodimer versus heterotetramer forms in H and F conformations (see Figure S4). From these results, we hypothesize that the Flipback conformation is important for association of cAMP with CBD-A. It is not known if RC heterodimers are important players in the activation mechanism of PKA, but if they are, we predict that the Flipback conformation plays a role in CBD-A association.

The only resolved conformation of full-length R in complex with C necessitated the R333K mutation to stabilize CBD-B.[11]

Scheme 1. Activation Mechanism of PKA RIα with Different R Conformations and Relative Association Rates



However, it has been shown that the R333K mutant (RC^{Holo}) has a solution structure different from that of WT. Small-angle X-ray scattering (SAXS) of the full-length WT heterodimer and heterotetramer[28] exhibit a shouldering region, one not observed in symmetric SAXS $p(r)$ distributions of mutant RC (R333K) and R_AC, a heterodimer lacking the B domain.[29]

The Flipback heterotetramer structure corroborates observations from multiple solution experiments. First, the C subunit interface of the WT full-length holoenzyme measured by HDXMS is consistent with the R/C interface of the $R_2C_2^{Flipback}$ structure, where the amides of residues $212–221^C$ and $278–289^C$ were reported to be unprotected.[14] Second, structural models from scattering experiments suggest that conformational changes in R cause the release of one set of R/C contacts,[30] consistent with the Flipback conformation (Scheme 1). It has been shown that binding of cAMP to CBD-B leads to an increase in the rate of H/D exchange at the B/C helix.[31] It is likely that once binding to CBD-B has occurred, the Flipback conformation is formed, resulting in CBD-A association. Attempts to elucidate the heterotetrameric structure of PKA RIα with SAXS have proposed a Flipback-like conformation.[28] Finally, the majority of R/C on RIα contact have been traced to interactions in CBD-A, consistent with Flipback.[10,14−16,21,29,32,33]

Flipback dynamics are consistent with other molecular simulations and models.[27,34] Guo and Zhou recently observed a flexible B/C helix when simulating the apo-B form,[9] the opposite of the starting point of our H form simulations. A backward-bending B/C helix is observed in these simulations, in a conformation resembling Flipback. This suggests that F is a state that can be accessed from both B and H forms.

An interesting role for Flipback emerges when we consider the termination phase of PKA regulation. The phosphodiesterase enzyme (PDE) hydrolyzes cAMP to 5′AMP, regulating concentration of the second messenger. Computational docking and HDXMS determined that for PDE to bind RIα subunit, the B/C helix requires a complete reorganization.[35] It is possible that Flipback is a binding partner of PDE, though further exanimation of this hypothesis is necessary.

Our simulations, coupled with experimental data, make the case for a viable and stable Flipback conformation of PKA RIα that may play important roles in the cAMP regulatory mechanism. Our work reveals a new structure of the WT PKA R subunit, which supports observations from ensemble-averaged solution structures and experiments. BD suggests a role for the $R^{Flipback}$ conformation in the mechanism of PKA activation. We hope our findings will lead to a re-examination PKA, especially with regard to differences between the conformations of WT and R333K mutants and the role of structural ensembles in ligand binding and, ultimately, signal transduction.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00461.

Detailed experimental methods, MD of the Flipback structure (Figure S1), the encounter complex (Figures S2 and S3 and Table S2), and electrostatic profiles of Holo and Flipback (Figure S4) (PDF)

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S.P.H. wrote the manuscript, analyzed MD, and performed and designed BD experiments. R.D.M. performed and designed MD experiments. R.E.A. directed the research.

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Notes

The authors declare the following competing financial interest(s): R.E.A. is a co-founder of, on the scientific advisory board of, and equity holder in Actavalon, Inc.

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■ ABBREVIATIONS

PKA, protein kinase A; cAMP, cyclic adenosine monophosphate; CBD, cyclic nucleotide binding domain; B, cAMP-Bound; H, Holo; F, Flipback.

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