A simple electrostatic switch important in the activation of type I protein kinase A by cyclic AMP

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Abstract

Cyclic AMP activates protein kinase A by binding to an inhibitory regulatory (R) subunit and releasing inhibition of the catalytic (C) subunit. Even though crystal structures of regulatory and catalytic subunits have been solved, the precise molecular mechanism by which cyclic AMP activates the kinase remains unknown. The dynamic properties of the cAMP binding domain in the absence of cAMP or C-subunit are also unknown. Here we report molecular-dynamics simulations and mutational studies of the RI α R-subunit that identify the C-helix as a highly dynamic switch which relays cAMP binding to the helical C-subunit binding regions. Furthermore, we identify an important salt bridge which links cAMP binding directly to the C-helix that is necessary for normal activation. Additional mutations show that a hydrophobic “hinge” region is not as critical for the cross-talk in PKA as it is in the homologous EPAC protein, illustrating how cAMP can control diverse functions using the evolutionarily conserved cAMP-binding domains.

Keywords: conformational changes; structure/function studies; molecular mechanics/dynamics; site-directed mutagenesis; ligand binding

Cyclic AMP (cAMP) is an extremely important and universal small molecule second messenger involved in the regulation of a myriad of cellular events (Antoni 2000). A conserved cAMP binding domain (Berman et al. 2005) is present in a large number of proteins,

including protein kinase A (PKA), EPAC, the cyclic-nucleotide gated channels, and cAMP-regulated bacterial transcription factors that mediate the allosteric effects of cAMP on protein functions. As cAMP analogs are increasingly being used as therapeutics, especially for cancer (Schwede et al. 2000; Kim et al. 2001), an understanding of the molecular features of activation may aid in the rational development of specific inhibitors and agonists that selectively target certain effector pathways.

Most of the biological effects of cAMP in eukaryotes are exerted through cAMP-dependent protein kinase, or PKA (Johnson et al. 2001). PKA has crucial roles in a large number of diverse processes such as learning and memory, apoptosis, and immune stimulation (Shabb 2001). In the absence of cAMP, two regulatory (R) and two catalytic (C) subunits form an inactive tetrameric holoenzyme complex. The R-subunits consist of two tandem cAMP-binding domains which each bind a molecule of cAMP, causing allosteric activation of the

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Abbreviations: PKA, protein kinase A or cyclic AMP-dependent protein kinase; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; ATP, adenosine triphosphate; C-subunit, catalytic subunit; R-subunit, regulatory subunit; PBC, phosphate binding cassette; CD, circular dichroism; K_a , activation constant; RMSD, root-mean-square distance; EPAC, exchange protein directly activated cAMP.

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C-subunit. Figure 1 shows the domain organization of the R-subunits. The N terminus is responsible for dimerization and subcellular localization of the R-subunits by A-kinase anchoring proteins. The C terminus consists of the two tandem cAMP-binding domains. In the middle is a variable linker region, which is thought to be largely disordered in solution (Li et al. 2000).

The crystal structures of the tandem cAMP binding regions of two isoforms of R-subunits, RI α (Su et al. 1995) and RII β (Diller et al. 2001), have been solved bound to cAMP. Each cAMP-binding domain consists of a contiguous eight-stranded β -barrel responsible for binding cAMP and a noncontiguous helical subdomain. Embedded in the β -barrel is the phosphate binding cassette (PBC), which is highly conserved and contains many of the residues that bind cAMP. In RI α , the C-terminal cAMP binding domain (B-domain) is responsible for regulating access of cAMP to the N-terminal cAMP-binding domain (A-domain), and cAMP binding to the A-domain

Figure 1. (Top) General domain organization of the regulatory subunits of protein kinase A. The A-domain is directly responsible for activating type I α protein kinase A in a cAMP dependent manner. The cAMP-binding B-domain, which is not in this simulation but is present in the crystal structure, regulates access of cAMP to domain A but does not directly participate in the conformational changes that activate the kinase. (Middle) Region used in the molecular dynamics simulations presented here. Residues 91–112, which include the pseudosubstrate inhibitor are present in the crystallized construct but are not resolved in this structure. (Bottom) The construct in which we experimentally tested the mutations described. This region consists of the A-domain and pseudosubstrate inhibitor. Shown as ribbon diagrams are the crystal structures of the 91–244 domain when cAMP-bound (*A*) and C subunit-bound (*B*; bound C subunit not shown). Phosphate-binding cassette (PBC) (yellow); C-helix (white).

causes activation of the holoenzyme. In the crystal structure, the N-terminal residues (92–112), which include the pseudosubstrate inhibitor site, are not seen and are presumed to be disordered. Deletion experiments of RI α revealed that residues 94–244 are sufficient for high affinity inhibition of the C-subunit in a cAMP-dependent manner (Huang and Taylor 1998). The crystal structure corresponding to this region is shown in Figure 1. This fragment of RI α (92–244) contains the pseudosubstrate-like inhibitor and most of cAMP binding domain A. High affinity binding to the C-subunit is achieved only when both the pseudosubstrate-like inhibitor (residues 94–98) and the C-helix (residues 236–244) are present.

A crystal structure of the RI α pseudosubstrate inhibitor and A-domain (92–244) bound to the C-subunit was recently solved (Kim et al. 2005). As expected, this structure shows the pseudosubstrate inhibitor bound to the active site of the C-subunit, and the C-helix of the A-domain of RI α bound to a peripheral region on the large lobe of the C-subunit. Unexpectedly, there are major conformational rearrangements in the helical region of the A-domain. In particular, the C-helix of RI α swings away from the PBC by almost 90° (Fig. 1). However, even upon comparison with the cAMP-bound structure, the molecular mechanism of cAMP activation is still unclear from the structure alone. These structures show the two end points: a cAMP-bound, C-subunit-free structure; and a cAMP-free, C-subunit-bound structure. However, there is no cAMP-free, C-subunit-free structure. Thus, it is not clear whether release of cAMP itself or binding of C is responsible for the large conformational changes. Thus, we initially focus our investigations on the conformational and dynamic changes that occur upon release of cAMP from the isolated R-subunit, independent of the C-subunit.

A mechanism for cAMP activation of PKA must somehow transmit a signal from the cAMP bound to site A to the distal helical region and/or the pseudosubstrate inhibitor region, in some way releasing its inhibition of the C-subunit. A recent structure of the RI α cAMP-binding domains has been solved with domain B lacking nucleotide. This structure shows that the C-terminal region acts as a hydrophobic cap, locking the adenine into the β -barrel when cAMP is present, and is released to an extended strand in the absence of cAMP (Wu et al. 2004a). A different activation mechanism was recently suggested from a comparison of the cAMP-free EPAC structure to the cAMP-bound PKA RI α subunit. The investigators speculate that removal of cAMP causes changes in a hydrophobic hinge region that connects the phosphate binding cassette (PBC) to the C-terminal helix, causing this helix to swing away from the PBC (Rehmann et al. 2003). However, no high-resolution structures of the same protein are available both

with and without cAMP, so care must be taken in interpreting these structures. Here, molecular dynamics simulations and site-directed mutants are used to further investigate the molecular basis for the cAMP-induced conformational changes in the RI α subunit.

Results

The RI α (91–244) construct contains the minimal motifs needed to tightly bind the C-subunit in a cAMP-dependent manner (Huang and Taylor 1998). This region contains both the cAMP-binding β -barrel as well as the helical region of the A-domain known to bind the C-subunit (Fig. 1). As residues 92–112 were not visible in this structure, they were not included in the simulations. Presented are two simulations: one in which cAMP is bound and one in which cAMP is removed. Based on these simulations and the EPAC study, four site-directed mutants (L203A, I204A, Y229A, and R241A) (Fig. 2) were constructed in the minimal RI α domain (91–244) to determine more precisely how cAMP activates PKA on a molecular level. Circular dichroism (CD) spectroscopy and thermal denaturation experiments are also performed on these mutants to determine global structural effects of each mutation.

There are no gross changes in secondary or tertiary structure for either simulation (RMSD from the original crystal structure remains <2.0 Å). Surprisingly, the

conserved phosphate binding cassette comprised of residues 199–210 retains its original hydrogen bonding network without cAMP (not shown). E200 and R209 are potential residues for the allosteric activation by cAMP since they bind the ribose and phosphate, respectively, and are absolutely conserved. E200 also forms a salt bridge with R241 in the helical subdomain, while R209 pairs with D170 in β -strand 3 of the β -barrel. Interestingly, when cAMP is present, the contact between E200 and R241 is broken early (Fig. 3B), and this allows a new salt bridge to be formed later in the simulation, between R239 and E143 (Fig. 3A), both in the helical subdomain. This is allowed by a movement of the C-helix, which contains both arginines, toward the other helices (Fig. 3D). Based on the full-length (A- and B-domains) crystal structure, Asp 267 of the A-helix of domain B contacts R241 and potentially stabilizes it. In addition, the hydrophobic capping of cAMP by Trp260 is also missing. Since we are lacking these contacts in our simulation, it is possible that the lack of these residues causes the C-helix swing that we see in the crystal structure. However, it is also possible that crystal contacts or contacts from the B-domain, which are present in the crystal structure but not our simulation, alter the equilibrium position of the C-helix such that the most favorable switch position we find is not seen in the crystal structure. Without cAMP, E200 flips away from the β -barrel (Fig. 3C) and interacts more strongly with R241, while the R239/E143 contact never forms. In both simulations, the salt bridge between R209 and E170 remains and thus appears to be cAMP-insensitive on the timescale on the simulation. However, the mechanism involving changes in the hydrophobic hinge region contacts adjacent to the PBC suggested by Rehmann et al. (2003) was not seen in our calculations. A portion of the crystal structure is shown in Figure 2, and structural comparisons between the end of the simulations with and without cAMP are shown in Figure 4. In contrast, the β -barrel remains relatively motionless in both simulations (the RMSD from the crystal structure never exceeds 1.0 Å). The major result from these simulations is that the C-helix is dynamic in that it is able to toggle between at least two different states: one associated with the PBC through an Arg 241–Glu 200 salt bridge, and one associated with the rest of the helical region.

It has been previously shown that the 91–244 construct of RI α is the minimal domain necessary to bind C-subunit with high affinity in a cAMP-dependent manner (Huang and Taylor 1998). This construct includes both the pseudosubstrate inhibitor and the cAMP-binding A-domain (Fig. 1). Thus, cAMP binding to the A-domain is directly responsible for activating the C-subunit while the B-domain serves to regulate access of cAMP to the A-domain (Herberg et al. 1996). Using the

Figure 2. Crystal structure of the RI α PBC, cAMP, and its interaction with the C-helix via Glu 200 and R241. Also shown are the three hydrophobic residues in the hinge region that we mutated to alanines. The cAMP is shown in yellow, while the B- and C-helices are white and the PBC is red. Dotted lines indicate hydrogen bonding.

Figure 3. Plots of selected distances and dihedral angles during the simulations. Cyclic AMP-bound simulation is shown in gray dots; simulation without cAMP is shown as a solid black line; crystal structure distances are shown as straight dotted lines. (A) Salt bridge distance between OE1 of E143 and HH22 of R239. This salt bridge is not present in the crystal structure or apo simulation, but it forms in the simulation with cAMP. (B) Salt bridge distance between OE2 of E200 and HH21 of R241. This contact is present in the crystal structure but it breaks early with cAMP present and never reforms. Without cAMP, this contact breaks but reforms immediately when E200 flips toward the helical subdomain. (C) The side-chain torsion angle of E200 (CA,CB,CG,CD). With cAMP, it remains stable due to hydrogen bonding to the 2'-OH of the cAMP. Without cAMP, E200 flips toward the helical subdomain in order to form a salt bridge with R241. (D) The distance of the C α atoms of E143 and R239. This represents the relative distances of the helices containing E143 and R239. The C helix containing R239 and R241 acts as a switch that moves toward the β -barrel without cAMP and moves toward the other helices when cAMP is present.

above simulation results and the Rehmann et al. (2003) study as a guide, we constructed four site-directed mutants (R241A, L203A, I204A, and Y229A) in the 91–244 construct instead of the full-length protein in order to focus on the activation process and not be distracted by the known cooperativity between the A- and the B- domains (Herberg et al. 1996). Locations of these residues are shown in Figure 2. R241A will abolish the salt bridge with E200, while the other three mutants will disrupt the hydrophobic packing shown in Rehmann et al. (2003) to be important in cAMP-mediated activation of EPAC and suggested by analogy to also be critical for cAMP activation of PKA.

We used a coupled kinase activity assay described by Cook et al. (1981) to monitor phosphorylation of the Kemptide peptide (LRRASLG) by the C-subunit, and thus inhibition of C-subunit by wild-type and mutant RI α (91–244) and activation of the holoenzyme by cAMP. The holoenzymes had very minimal kinase

activity, showing that in the absence of cAMP, C-subunit binding and inhibition were not severely disrupted for any of the mutants. As shown in Figure 5 and Table 1, we measure the activation constant (K_a) for wild type to be $0.9 \pm 0.1 \mu\text{M}$, comparable to that determined previously (Huang and Taylor 1998). However, the R241A mutant has a $K_a \sim 10$ -fold higher, showing that its activation by cAMP is severely disrupted compared to wild type. The I204A mutant also shows a K_a slightly higher than wild type ($2.4 \pm 0.4 \mu\text{M}$), L203A show no significant different from wild type, while Y229A shows a slightly lower K_a ($0.66 \pm 0.1 \mu\text{M}$) than wild type.

The hydrophobic hinge (especially L203 and Y229) is well-conserved among the cAMP-binding domains (Canaves and Taylor 2002), but mutating these residues does not cause large defects in the activation of the C-subunit, as predicted by Rehmann et al. (2003). To determine if these residues are evolutionarily conserved

Figure 4. End structures of the cAMP-bound and cAMP-free simulations. Shown in ribbons are the A-, B-, and C-helices. The white ribbons represent the end of the cAMP-free simulations, where the E200 flips away from the PBC and interacts more strongly with R241. In green is the end of the cAMP-bound simulations, where the R241/E200 contact is broken and the R239 interacts with E143 of the A-helix. While we do not expect the details of these simulations to be fully accurate, they demonstrate that the R241/E200 salt bridge is able to report on the occupancy of cAMP and thus allow a direct allosteric connection with the C-binding helices, via the C-helix. The orange arrows represent the movement of the C-helix. Figure courtesy of Nina Haste.

in order to provide structural stability to the domain, we obtained circular dichroism spectra and thermal melting profiles of the cAMP-free proteins at 222 nm. CD spectra show significant α -helical content with minima at 208 and 222 nm, as expected. Mutant spectra are fully superimposable with wild type (not shown), demonstrating that the mutations do not cause severe structural defects. However, the R241A and Y229A mutants unfold with a melting temperature (T_m) $\sim 10^\circ\text{C}$ lower than wild type (Fig. 6, Table 1), while the I204A mutant unfolds $\sim 3^\circ\text{C}$ lower. Interestingly, the T_m of the L203A mutant is slightly higher to that of wild type, but it unfolds much less cooperatively. The folding was irreversible due to the protein precipitating at the higher temperatures, so we could not extract thermodynamic parameters.

Discussion

Before we discuss the implications of this study, it is important to clarify why the 91–244 construct of RI α is an appropriate model for evaluating specific features of the activation process. It has been previously shown that the hydrophobic capping of the adenine ring of the

cAMP is a major determinant of regulation in all cAMP-binding domains (Berman et al. 2005), especially by coupling binding of cAMP to interactions with other proteins and to DNA in the case of CAP. The minimalist 91–244 piece of RI α lacks the entire B-domain, including the hydrophobic capping residue (W260) as well as D267, which in the crystal structure of the isolated cAMP-binding domain's crystal structure interacts with the R241/E200 salt bridge. However, this construct still retains high-affinity cAMP binding and C-subunit binding (Ringheim and Taylor 1990; Huang and Taylor 1998). Binding of cAMP is ~ 10 -fold weaker than full-length (Ringheim and Taylor 1990), presumably because the hydrophobic capping is missing. Importantly, the activation constant (K_a) of this construct is ~ 10 -fold higher than full-length, mirroring the increased K_d for cAMP binding. This shows that although the hydrophobic capping that is crucial for cross-talk between the A- and the B-domains is missing, full allosteric cAMP-induced activation is retained in this fragment. It is our goal here to uncover the regulation mechanisms independent of hydrophobic capping that are crucial in type I α PKA activation by using this minimalist segment.

The molecular dynamics simulations importantly demonstrate the stable nature of the β -barrel subdomain compared to the C-helix, at least on the timescales used. These simulations were performed before the crystal structure of the RI α (A-domain)/C complex, which confirmed the malleability of the C-helix and the stability of the β -barrel. Importantly, the simulations suggest that even in the absence of the C-subunit, the C-helix has an intrinsic dynamic nature that is crucial for the

Figure 5. Activation of wild-type and mutant RI α (91–244)/C holoenzymes. Holoenzymes were prepared as described in Materials and Methods. Holoenzymes (30 nM) were incubated with various concentrations of cAMP, and the activity was measured as described previously (Cook et al. 1981). Symbols used are wild type, $\square$; I204A, $\blacksquare$; R241A, $\triangle$. The activation profiles for the L203A and Y229A mutants are very similar to those of wild type and are omitted for ease of viewing, but their activation constants are listed in Table 1.

Table 1. Activation constants and melting temperatures of mutants

	K_a (μ M)	T_m ($^{\circ}$ C)
WT 91-244	$0.9 \pm .1$	47 ± 1
R241A	9.1 ± 1	38 ± 1
L203A	$0.8 \pm .1$	50 ± 2
I204A	$2.4 \pm .4$	44 ± 1
Y229A	$0.66 \pm .1$	38 ± 1
WT full-length ^a	$0.14 \pm .01$	

Activation constants are of the wild-type and mutant RI α (91–244)/C holoenzymes determined from Figure 5. The melting temperatures are for the cAMP-free wild-type and mutant RI α (91–244) subunits, as determined from Figure 6.

^aThe K_a values for full-length WT are taken from Huang and Taylor (1998).

C-subunit-induced conformational change during the activation/inactivation process. We showed that this dynamic nature is modulated by the presence of cAMP, providing a potential molecular mechanism for activation. Furthermore, our mutation of Arg 241, which modulates the interaction of the C-helix with the cAMP-binding PBC through Glu 200, showed a significant activation deficiency consistent with the simulations. Although the presence of the B-domain will undoubtedly affect the specific interactions, the basic idea of C-helix mobility in the activation process will remain in the full-length protein. Importantly, mutation of R241 in the full-length protein causes a similar activation deficiency of ~ 10 -fold (Steinberg et al. 1991). The importance of a dynamically regulated C-helix is likely to be conserved in all cAMP-binding domains, although the specific ways it is regulated will vary.

The importance of the E200/R241 salt bridge in the activation mechanism is supported by the inability of cAMP analogs lacking the 2'-OH to activate the type I α holoenzyme (Schwede et al. 2000). Mutations of E200 and R241 are a natural way to further test the importance of the E200/R241 salt bridge in activation of holoenzyme. However, mutation of E200 completely abolishes cAMP binding and thus cannot be used to test the importance of this residue in activation (Steinberg et al. 1991). Mutations of R241 in the full-length protein caused deficiencies in holoenzyme activation, but the investigators ascribed this effect largely to disrupted communication between the A and the B-domains (Symcox et al. 1994). However, our mutation of this residue within the minimal construct shows a similarly large activation deficiency (Table 1) and thus implicates the E200/R241 salt bridge as a positive factor in the direct coupling of cAMP that is disrupted when C-subunit binds to the A-domain of RI α . While our study implicates the E200/R241 salt bridge as part of the activation mechanism of PKA by cAMP, there must be

other important allosteric determinants as well since the R241A mutant does not completely uncouple cAMP and C-subunit binding. R209 mutants have been previously shown to be partially deficient in activation (Bubis et al. 1988). Also, the Rp-cAMP analog, which has a sulfur in place of the exocyclic oxygen that binds R209 (Wu et al. 2004b), serves as an antagonist of cAMP, able to bind the R-subunit but not able to activate PKA (Dostmann et al. 1990). D170 forms a salt bridge with R209 and also appears to play an important role when C-subunit is bound. We propose that in PKA, the E200/R241 and R209/D170 salt bridges synergize to allow the allosteric activation of C by cAMP. The hydrophobic hinge region may undergo conformational changes upon release of cAMP and binding of C-subunit, but we have shown here that sequence-specific contacts are not required for the allostery as they are in EPAC.

In RII β , the equivalent of E200 forms a salt bridge with an arginine from the B-domain, while the equivalent of R241 only forms a salt bridge with the equivalent of D267 in the B-domain. The B-domains of both RI α and RII β also lack the Arg/Glu salt bridge that we see as important in the A-domain of RI α . In each case, the equivalent of E200 interacts with a charged residue that is linked to the hydrophobic capping motif. In the context of full-length RI α , the C-helix is also regulated by the B-domain, via W260 capping of cAMP and D267 binding to R241. In addition, the residue analogous to RI α Glu 200 in EPAC is a Gln; thus there is no analogous salt bridge in EPAC that is important in its activation mechanism. Therefore, the specific R241/E200 salt bridge is crucial for the A-domain of RI α but is not an essential requirement for all cAMP-binding domains.

Figure 6. Thermal denaturation profiles of cAMP-free wild-type and mutant RI α (91–244). Samples (0.1 mg/mL) were scanned at 20° C/h from 20° – 80° C using a 20-sec integration time. Ellipticity was monitored at 222 nm. Symbols used are WT, $\square$; I204A, $\blacksquare$; R241A, $\triangle$; L203A, $\bullet$; Y229A, $\blacktriangledown$.

The thermal denaturation studies show that all three hydrophobic residues, Y229 in particular, are important for the stability of the domain, serving as a nucleus to connect the two subdomains. This is the likely reason these residues are conserved. As we are monitoring the unfolding at 222 nm, we are mostly monitoring the folding of the helical subdomain instead of the β -barrel. Thus, these mutants somehow affect the packing of the helices at higher temperatures but do not alter the gross structure at room temperature. Y229 resides in the middle of the helical subdomain and serves to connect the various helices, so its replacement with alanine likely leads to sub-optimal helical packing. I204 and L203, on the other hand, reside at the interface between the helical and β -barrel subdomains, so their replacement with alanine would not necessarily be expected to affect the helical folding as much as Y229. Whether the two subdomains fold cooperatively or independently is not clear, so it is possible that I204 and L203 affect the coupled folding between the two. The hydrophobic hinge is likely critical in allowing the C-helix to swing out, but our mutants show that it is the general hydrophobic character and not a specific three-dimensional arrangement that is important for this. On the other hand, the added structural stability imposed by a specific three-dimensional arrangement of specific hydrophobic residues could be reason enough to conserve these residues, even if they do not change the mechanism of cAMP activation. Interestingly, there is no correlation between thermal stability and activation constants of the hydrophobic mutants, further evidence that their roles in linking the two subdomains provide a structural rather than mechanistic role.

The crystal structure of EPAC in a cAMP-free form combined with mutations identified a hydrophobic hinge region by which cAMP is coupled to guanine nucleotide exchange. The equivalents of Y229 and L203 in EPAC form a hydrophobic patch that allows the C-helix to swing outward upon release of cAMP. Due to the high sequence conservation between PKA and EPAC, it was suggested that the same residues are likely to couple cAMP to C-subunit activation in PKA. While our Y229A mutant shows a slightly lower K_a than wild type as in EPAC, the effect is not as large as seen for EPAC. However, our L203A mutant shows no significant difference in its activation. Our mutation of I204, which is part of the same hydrophobic hinge, shows a K_a almost three times higher than wild type, but is still much smaller than the 10-fold difference seen for the R241A mutant. Importantly, the EPAC study clearly demonstrated the importance of the C-helix in propagating the cAMP signal. Likewise, a crystal structure of the cAMP-activated potassium channel showed a similarly large outward movement of the C-helix upon binding cAMP, suggesting its importance in the activation process (Clayton et al. 2004). Together

with the R/C complex crystal structure we confirm for PKA that changes in the C-helix are important for the activation of PKA. The specifics of regulation of the C-helix by cAMP is likely to be very different for each cAMP binding domain, and we show here that for type I PKA, a salt bridge is crucial.

The crystal structure of the R/C complex demonstrates that the totality of the conformational changes between two end states: the free, cAMP-bound structure, and the cAMP-free, C-subunit-bound structure, including a large outward swinging of the C-helix. These structures, however, do not provide information on the dynamic properties affected by cAMP. Specifically, it is not entirely clear from the structure alone how cAMP causes the allosteric changes necessary to disrupt interactions with the C-subunit. Nor is it clear whether the simple release of cAMP alone would be sufficient to generate a more extended C-helix. In addition to implicating a specific salt bridge, our study here provides evidence that although cAMP release alone may not cause large conformational changes, it causes changes in dynamics that are central to the proper recognition of the C-subunit. However, the release of cAMP alone is not sufficient to mediate the major change in conformation that was observed in the R/C complex structure, at least on the time-scale of our simulations. It is thus C-subunit binding that then causes a large conformational change. This is in agreement with studies on the bacterial CAP transcription factor, which have also demonstrated a change in global dynamics upon cAMP binding, in the absence of significant secondary structure changes (Dong et al. 2002).

Our study clearly implicates the dynamic nature of the C-helix in the activation process, which is likely present in all cAMP binding domains, and identifies a clear ionic switch mechanism of the C-helix that is isoform specific. This information adds to the known effect of hydrophobic capping in the regulation process. In this case, at least for this small construct, hydrophobic capping is not necessary for the mechanics of activation. A full determination of other mechanisms that in concert with the hydrophobic capping affect the position of the C-helix in other cAMP-binding proteins will lead to a clearer picture of how evolution has used this basic binding module to affect cAMP signaling in diverse ways. This study also highlights how molecular simulations can be useful for the generation of specific hypotheses about how small molecules participate in the allosteric activation of signaling processes.

Materials and methods

Protein structure

The Protein Kinase A regulatory subunit structure was taken from the Protein Data Bank (1RGS; Su et al. 1995). Only domain

A (residues 113–244) was used in the simulations. For the cAMP-free simulation, the cAMP molecule was simply removed from this structure. Missing side-chain atoms of six residues were incorporated with Insight (MSI) using standard geometries.

Simulation protocol and setup

Two molecular dynamics simulations were conducted using the SANDER module in AMBER 6.0 (University of California, San Francisco) with the standard Cornell et al. (1995) force field. Hydrogens were added to the structures, and preparation of the topology and parameter files was done with the AMBER suite of programs. Partial charges and other simulation parameters for the cAMP molecule were taken from the QUANTA/CHARMM nucleic acids database (Brooks et al. 1983). Na^+ and Cl^- counterions were placed around the proteins to ensure neutrality and to mimic physiological salt. TIP3P water molecules were placed to form a rectangular box, with sides no closer than 10 Å from the surface of the protein. The cAMP-bound simulation contained 8188 TIP3P waters with box dimensions $70 \times 63 \times 59$ Å, while the cAMP-free simulation contained 8191 TIP3P waters with box dimensions of $73 \times 68 \times 55$ Å. Periodic boundary conditions with constant temperature and pressure were used. Temperature was kept at 300 K by coupling to an external heat bath using the Berendsen algorithm (Berendsen et al. 1984). Pressure was kept at 1 atm by isotropic scaling using a Berendsen barostat. Particle Mesh Ewald with cubic spline approximation was used to treat long-range electrostatics (Essmann et al. 1995). The SHAKE algorithm (Ryckaert et al. 1977) was used to constrain covalent bonds to hydrogen so that a time step of 2 fsec could be used. Structures were energy minimized by steepest descent for 500 steps in vacuum prior to adding counterions or waters. After counterions and waters were added, 500 more steps of steepest descent energy minimization were applied. Then, the waters were allowed to equilibrate for 30 psec while the protein was held fixed. The entire system was then allowed to further equilibrate for 30 psec by raising the temperature slowly to 300 K. After a total of 500 psec of further equilibration, coordinates were saved every 1 psec for analysis, with the cAMP-free simulation lasting 4.5 nsec and the cAMP-bound simulation lasting 3 nsec. Simulations were performed at NCSA and the San Diego Supercomputer Center.

Trajectory analysis

Secondary structure analysis was performed with the DSSP program (Kabsch and Sander 1983). Atomic distances and side-chain torsion angles were calculated using the PTRAJ module within the AMBER software. RMSD values were calculated from the starting crystal structure using the PTRAJ module.

Site-directed mutagenesis

Site-directed mutants (R241A, L203A, I204A, and Y229A) within the pRSET (91–244) bovine RI α construct were prepared with QuikChange II kits (Stratagene) using standard procedures. Briefly, complimentary oligonucleotide primers containing the desired mutations were constructed (Allele Biotechnology). The primers were then incubated with the template plasmid, dNTPs, and Pfu Turbo Polymerase to extend the primers for 18 cycles of thermal cycling. Following the

amplification, the methylated template DNA was incubated with DpnI for 1 h at 37°C, and the product was transformed into XL-1 Blue *Escherichia coli* supercompetent cells. All of the above reagents, including the competent cells, are from the QuikChange II kit. Plasmid DNA was then purified from the XL-1 cells using QiaPrep Miniprep (Qiagen). Mutant sequences were confirmed by DNA sequencing (UCSD Cancer Center Core).

Protein expression and purification

Wild-type and mutant RI α (91–244) were expressed in *E. coli* BL-21 DE3. Cells were grown in ampicillin-containing LB media at 37°C to an absorbance of 0.6 at 600 nm, and then induced with IPTG for an additional 6 h at 25°C. Cells were then centrifuged, lysed with a French press, and centrifuged again to remove the particulate fraction. Protein was then precipitated using ammonium sulfate at 45% of saturation. cAMP-Sepharose resin was used to affinity-purify the proteins. One hundred micromoles (100 μmol) of 8-AEA cAMP (Biolog) was coupled to 25 mL of NHS-activated Sepharose fast flow (Amersham Biosciences) using the protocol supplied by the vendor. Five milliliters (5 mL) of resin was used per 2 L of cell culture. Three batch elutions (7 mL each) were performed at room temperature for 30 min each with buffer containing 25 mM cGMP (Sigma), 20 mM MES, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, and 5 mM DTT (pH 5.6). Eluted fractions were pooled and then further purified using gel filtration (Superdex 200) in a buffer containing 50 mM MES, 200 mM NaCl, 2 mM EDTA, 2 mM EGTA, and 5 mM DTT. Recombinant C-subunit was expressed and purified as previously described (Slice and Taylor 1989). Heterodimers were then formed by mixing purified R-subunit with a molar excess of purified C-subunit and dialyzing overnight against a buffer containing 50 mM MOPS, 150 mM NaCl, 2 mM MgCl_2 , and 0.2 mM ATP (pH 7.0). These conditions approximate physiological concentrations for Mg^{2+} /ATP for most tissues. Gel filtration (Superdex 75) was then used to separate heterodimers from free C-subunit.

Holoenzyme activation

Protein kinase activity was assayed spectrophotometrically with a coupled kinase activity (Cook et al. 1981) using Kemptide (LRRASLG), which was synthesized by the Microchemical Facility of the University of California, Berkeley. The oxidation of NADH, monitored spectrophotometrically as an absorbance decrease at 340 nm, is coupled to the production of ADP by lactate dehydrogenase and pyruvate kinase. Holoenzymes at concentrations of 30 nM were incubated for 5 min in the assay mix (500 μL of holoenzyme in above buffer, with 1 mM phosphoenolpyruvate, 0.3 mM NADH, 12 units of lactate dehydrogenase, and 4 units of pyruvate kinase, with varying concentrations of cAMP (Sigma) ranging from 2 nM to 50 μM). Reactions were carried out in a disposable 1-mL cuvette. The reaction was initiated by adding Kemptide (100 μM) and the activity of the free C-subunit was followed using the spectrophotometric assay. Three replicates for each reaction were performed. Nonlinear regression using the Graphpad Prism software was used to determine the activation constant (K_a) for wild type and mutant holoenzymes.

Circular dichroism

For all measurements, wild type and mutant RI α (91–244) were dialyzed into a buffer containing 25 mM sodium phosphate, 150 mM NaCl, 0.5 mM EDTA (pH 7.0). Measurements were performed on an AVIV 202 CD spectropolarimeter using a 0.2-cm path-length microcuvette. CD spectra were scanned at 25°C from 255 nm to 195 nm at 0.5 nm resolution and an integration time of 20 sec. For thermal unfolding curves, samples were scanned at 222 nm at 20°C/h from 20–85°C using a 20-sec integration time. Each measurement was performed in triplicate and deviations between scans were negligible. Baseline subtraction was performed by the AVIV CDS program.

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