Cyclic AMP Analog Blocks Kinase Activation by Stabilizing Inactive Conformation: Conformational Selection Highlights a New Concept in Allosteric Inhibitor Design*

Suguna Badireddy‡, Gao Yunfeng‡, Mark Ritchie§, Pearl Akamine∥, Jian Wu∥, Choel W. Kim¶, Susan S. Taylor¶, Lin Qingsong‡, Kunchithapadam Swaminathan‡**, and Ganesh S. Anand‡**

The regulatory (R) subunit of protein kinase A serves to modulate the activity of protein kinase A in a cAMP-dependent manner and exists in two distinct and structurally dissimilar, end point cAMP-bound “B” and C-subunit-bound “H”-conformations. Here we report mechanistic details of cAMP action as yet unknown through a unique approach combining x-ray crystallography with structural proteomics approaches, amide hydrogen/deuterium exchange and ion mobility mass spectrometry, applied to the study of a stereospecific cAMP phosphorothioate analog and antagonist((R)p-cAMPS). X-ray crystallography shows cAMP-bound R-subunit in the B form but surprisingly the antagonist Rp-cAMPS-bound R-subunit crystalized in the H conformation, which was previously assumed to be induced only by C-subunit-binding. Apo R-subunit crystalized in the B form as well but amide exchange mass spectrometry showed large differences between apo, agonist and antagonist-bound states of the R-subunit. Further ion mobility reveals the apo R-subunit as an ensemble of multiple conformations with collisional cross-sectional areas spanning both the agonist and antagonist-bound states. Thus contrary to earlier studies that explained the basis for cAMP action through “induced fit” alone, we report evidence for conformational selection, where the ligand-free apo form of the R-subunit exists as an ensemble of both B and H conformations. Although cAMP preferentially binds the B conformation, Rp-cAMPS interestingly binds the H conformation. This reveals the unique importance of the equatorial oxygen of the cyclic phosphate in mediating conformational transitions from H to B forms highlighting a novel approach for rational structure-based drug design. Ideal inhibitors such as Rp-cAMPS are those that preferentially “select” inactive conformations of target proteins by satisfying all “binding” constraints alone without inducing conformational changes necessary for activation. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.004390, 1–14, 2011.

cAMP is an important second messenger that allows both prokaryotes and eukaryotes to sense cues from the environment and then translate extracellular signals into a biological response (1). The cyclic nucleotide binding (CNB)¹ domain is an ancient motif that appears to have co-evolved as the primary receptor for cAMP (2). CNBs function as regulatory modules in different classes of proteins, such as catabolite activator protein, cyclic nucleotide gated channels, Epac proteins, and guanine-nucleotide-exchange factors, where effector protein and domain activity is controlled by cAMP binding to CNB (3). In eukaryotes, one of the principal targets of cAMP is the cAMP-dependent protein kinase, protein kinase A (PKA), whose regulatory subunit (R-subunit) contains two tandem CNBs. In the absence of cAMP, PKA exists as an inactive tetrameric holoenzyme with the homodimeric R-subunit exist as an ensemble of both B and H conformations. Although cAMP preferentially binds the B conformation, Rp-cAMPS interestingly binds the H conformation. This reveals the unique importance of the equatorial oxygen of the cyclic phosphate in mediating conformational transitions from H to B forms highlighting a novel approach for rational structure-based drug design. Ideal inhibitors such as Rp-cAMPS are those that preferentially “select” inactive conformations of target proteins by satisfying all “binding” constraints alone without inducing conformational changes necessary for activation. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.004390, 1–14, 2011.

Molecular & Cellular Proteomics 10.3 10.1074/mcp.M110.004390–1

Want to cite this article? Please look on the last page for the proper citation format.

The abbreviations used are: CNB, cyclic nucleotide binding; cAMP, Cyclic adenosine 3’,5’-monophosphate; ESI QTOF, Electrospray ionization Quadrupole Time-of-flight; PKA, protein kinase A; H/D, hydrogen/deuterium; PBC, phosphate binding cassette; CCS, collisional cross-sectional area.

© 2011 by The American Society for Biochemistry and Molecular Biology, Inc. This paper is available online at http://www.mcponline.org
across all mammalian tissue and are the best studied iso-
foms of PKA (5). Of the two CNBs (CNB-A and CNB-B) in RI
/H9251,
the N-terminal CNB-A, together with a pseudo substrate re-
gion (IS) (6) and corresponding to the deletion fragment,
RI/H9251 (91–244), is sufficient for mediating high-affinity interac-
tions with both cAMP and the C-subunit (7) (Fig. 1)
A). The most
critical interaction sites for the C-subunit are localized entirely
within this fragment (8). Although regions N-terminal to the
pseudosubstrate site have been predicted to associate
weakly with the C-subunit on the basis of limited proteolysis
footprinting experiments (9), there were no significant differ-
ences in amide hydrogen/deuterium (H/D) exchange that
could be attributable to regions N-terminal to the pseudosub-
strate site between the full-length apo RI
/H9251 and RI
/H9251: C (10)
complexes. Furthermore comparisons of the amide H/D ex-
change between apo and cAMP-bound RI
/H9251 showed no
differences in the N-terminal region of the protein (10). RI
/H9251(91–
244) thus serves as a generic prototype for the CNB domain
as well as a minimal model for mapping interactions with both
cAMP as well as the C-subunit, and has been analyzed com-
prehensively in solution using amide H/D exchange mass
spectrometry (11) and nuclear magnetic resonance spectros-
copy (NMR) (12).

The structural biology of CNBs from each of the above
classes of molecules reveals a highly conserved architecture
with two subdomains: a \( \beta \)-subdomain with an eight-stranded
\( \beta \)-sheet, containing a solvent-shielded pocket for binding
cAMP and a noncontiguous \( \alpha \)-subdomain. The structure of
the PKA holoenzyme complex (8, 13) revealed a dramatic
conformational change in the R-subunit compared with the
cAMP-bound form and was termed the “H-conformation” (Fig.
1B). Structures of the CNB domain have been solved with the
ligand cAMP (14, 15) and this cAMP-bound conformation has
been denoted the “B-conformation” (Fig. 1C). A comparison

---

**Fig. 1. Conformational dynamics of the PKA R-subunit and cAMP-dependent regulation of PKA.**

A, Domain organization of PKA RI\(\alpha\). An N-terminal dimerization/docking domain (D/D (gray)) is followed by an inhibitory region/pseudo substrate region (IS, red) and two tandem cAMP binding domains, CNB-A and CNB-B at the C-terminal end. A deletion mutant, RI\(\alpha\) (74–379) (RAB) with IS, CNB-A and CNB-B. A smaller deletion mutant containing IS and CNB-A alone, RI\(\alpha\) (91–244) (RA), is sufficient for high affinity binding to the C-subunit(7). B, Structure of the R-subunit in the C-subunit-bound conformation (H-conformation) (from the R\(\alpha\): C complex structure, PDB: 3FHI). The \( \alpha \)-subdomain is in pale green and the \( \beta \)-subdomain is in tan. C, Structure of the R-subunit (bound to cAMP, PDB: 1RGS) in the B-conformation. D, Apo R-subunit toggles between cAMP-bound and C-subunit-bound states. E, The width of the Phosphate Binding Cassette (PBC) pocket in the H form is 10.1 Å (between the C\(O\) atoms of Leu 201 and Pro 208). F, The corresponding width of the PBC pocket in B form is 8.7 Å. cAMP binds with high affinity to the pocket with two critical and conserved amino acids, Glu 200 and Arg 209, which coordinate binding to the ribose 2'-OH and the equatorial oxygen of cAMP, respectively. Asp 170, present in the \( \beta 1-\beta 2 \) loop, is seen forming a salt bridge with Arg 209, which is critical for allosteric communication between the PBC and the C-subunit-interface. All the above figures were generated with the program pyMOL.
of the structures of the B and H forms demonstrated for the first time that the CNB domain has a remarkable dynamic range in terms of the conformations it can assume. This established the R-subunit as a conformational toggle switch between B (14, 15) and H (8) conformational states (Fig. 1D). The phosphate binding cassette (PBC) within the β sub-domain reveals a conserved hydrogen bonding network, connecting residues that are critical for specific binding to cAMP (3). This presumably provides a solvent-shielded and phosphodiesterase-resistant environment for cAMP. One of these residues is Arg 209 (in PKA, CNB-A), which is positioned with precise geometry to anchor the equatorial oxygen of the critical phosphodiester bond of cAMP (Figs. 1E, 1F). A second residue is Glu 200, which is within H-bonding distance of the 2’OH of the ribose moiety. Substitutions at both these residues abolish cAMP binding to the PKA R-subunits (16, 17).

One of the major puzzles in the field of cAMP signaling is that out of numerous cAMP analogs screened, only the R-enantiomers of thio-substituted cAMP analogs or phosphoro-thioates (R-pcAMPS and abbreviated to Rp), where the equatorial oxygen of cAMP is substituted with sulfur) have been observed to be antagonists of PKA (17, 18). Recent solution studies of Rp bound to PKA indicate that it functions more as an inverse agonist to increase intersubunit interactions via increased dynamics of each of the subunits (19). In contrast, substitution of the oxygen at the axial position with sulfur yields the cAMP mimetic and agonist Sp-cAMPS (abbreviated to Sp). In this study, we have used a combination of x-ray crystallography and solution mass spectrometry (solution phase amide H/D exchange and ion mobility mass spectrometry) to describe the structure and conformational dynamics of the R-subunit by comparing the apo, cAMP-bound, the agonist Sp and inverse agonist Rp-bound states. In addition to establishing the molecular basis for antagonism of Rp, our results address the basis for the 1000-fold difference in affinity between the cAMP-bound and apo R-subunit for the C-subunit (20).

Because Rlα(91–244) containing CNB-A is a versatile minimal model for examining cAMP as well as C-subunit interactions and conformational changes, we have used this double deletion construct in this study, henceforth referred to as RA. Our results reveal that the apo and cAMP-bound R-subunit crystallized in the B-conformation and the two structures are nearly superimposable. Surprisingly, the Rp-bound RA crystallized in the H-form, in the same conformation as the inactive RA:C holoenzyme complex (8). Our results show that whereas x-ray crystallography reveals a snapshot of the apo Rα−subunit in the B conformation, an analysis of temperature factors, amide deuterium exchange measurements and collisional cross sectional areas of molecules from ion mobility mass spectrometry indicate that the R-subunit is highly dynamic and exists as an ensemble of at least two endpoint B (15) and H (8) conformations. It is clearly evident that cAMP and a cAMP-agonist (Sp) preferentially recognize and bind the B conformation, whereas the C-subunit and the cAMP-agonist (Rp) preferentially recognize and bind the H conformation. We propose this represents “conformational selection” in apo Rlα. In contrast, interconversion of cAMP-bound B-form and C-subunit-bound H-form likely occurs via “induced fit.” Our study provides valuable insights into the allosteric regulation of PKA by cAMP and has important implications for designing small molecule disruptors of protein-protein interactions.

**MATERIALS AND METHODS**

**Materials—**BL21 (DE3) E. coli strains were purchased from Novagen (Madison, WI). TALON metal affinity resin was from Clontech laboratories (Mountain View, CA). 8-aminohexyl adenosine-cAMP, 8-Bromoadenosine-3’-5’ cyclic monophosphorothioate, Rp-isomer, cAMP, cGMP and 8-Bromoadenosine-3’-5’ cyclic monophosphorothioate, Sp-isomer were from Bredin Life Science Institute (Bremen, Germany). 5'-adenylylimidophosphate (AMP-PNP) was from Sigma-Aldrich (Singapore). Porosyme-immobilized Bepsin carboxylase was from Applied Biosystems (Foster city, CA). Deuterium oxide (D2O) and protein sequence analysis grade trifluoroacetic acid were from Fluks BioChemi (Buchs, Switzerland). Crystal plates, crystallization screens were from Hampton Research (Aliso Viejo, CA) and Jena Bioscience GmbH (Jena, Germany). All other reagents were reagent grade.

**Expression and purification of PKA C-subunit—**The PKA C-subunit with an N-terminal hexahistidine tag was expressed in E. coli [BL21 (DE3)] and purified using the Talon resin. Large scale expression was achieved by culturing bacteria at 37 °C until mid exponential phase, followed by induction with 500 μM IPTG overnight at 20 °C. Cells were harvested at 6000 × g (Beckman Coulter JA-10 rotor) for 20 min and the cell pellet was resuspended in lysis buffer [50 mM potassium monobasic phosphate, 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM β-mercaptoethanol, 5 mM imidazole]. Cells were lysed by a sonicator (Misonix) and centrifuged at 17,000 × g (Sigma, Sartorius, 19776-H rotor) at 4 °C for 40 min and the supernatant was incubated with talon resin at 4 °C for 1 h. The resin was then transferred into columns (Bio Rad). Washes were performed with both lysis buffer and wash buffer (Lysis buffer, pH 7.5) followed by elution buffer containing lysis buffer with 200 mM imidazole, pH 7.0. Further purification was achieved by size-exclusion chromatography [S200 column, AKTA system (GE Healthcare)].

**Expression and Purification of PKA Rα—**PKA Rα was expressed in E. coli [BL21 (DE3)] and purified using cAMP Sepharose affinity chromatography as described earlier (14). Cells, grown up to mid-exponential phase, were induced with 500 μM IPTG overnight at 20 °C. Cells were harvested at 6000 × g (Beckman Coulter JA-10 rotor) for 20 min and the cell pellet was resuspended in lysis buffer (20 mM 2-N-(morpholino)ethanesulfonic acid pH 6.5, 100 mM NaCl, and 2 mM EDTA) and lysed by sonication. Centrifugation of cell lysates was carried out at 17,000 × g for 40 min and the supernatant was precipitated with 45% ammonium sulfate. The ammonium sulfate precipitate was separated from supernatant by centrifugation at 6500 × g for 15 min and resuspended in lysis buffer followed by incubation with cAMP Sepharose resin overnight at 4 °C. The resin was then transferred into columns and purified Rα was eluted with 40 mM cGMP (50 mM 2-N-(morpholino)ethanesulfonic acid pH 5.8, 200 mM NaCl, 2 mM EDTA, 40 mM cGMP). The protein was further purified by size-exclusion chromatography [S75 column, AKTA (GE Healthcare)].

**Purification of PKA Holoenzyme—**Rα and C-subunit in a 3:1 molar ratio were dialyzed for 16 h, against buffer containing 10 mM Mops
Crystal screens were performed at room temperature with Hampton and JB screens by the hanging drop vapor diffusion method. Reproducible crystals were obtained after 3 days in the JB 6 (D6) condition (100 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5), 2 M ammonium sulfate). Crystallization of RA:C holoenzyme in the presence of 0.2 mM AMP-PNP and Rp generated crystals of RA-Apo bound to Rp alone. In both cases there was no trace of the C-subunit in the crystal. We again suspect the high salt in the crystallization conditions resulted in dissociation of the C-subunit. Prior to data collection, crystals were briefly soaked in a cryoprotectant consisting of paratone and mineral oil (1:1 ratio) and flash frozen in liquid nitrogen. A complete dataset was collected on a Bruker Platinum 135 CCD detector mounted on a Bruker MicrostarH x-ray generator. The dataset was processed and scaled using the HKL2000 program (25). Matthew’s coefficient confirmed that the crystal was that of Rp-bound RA and not that of Rp-bound to the RA:C holoenzyme. The presence of R (and not cAMP or cGMP) was unequivocally confirmed, based on bromine density at the C8 position of Rp (Fig. 3). The structure was solved by molecular replacement, using the Molrep program (21) and the RA coordinates of the holoenzyme complex from the PDB file 3FHI (8) as the search model. Interestingly, the coordinates of the holoenzyme complex gave better solutions than when the coordinates of cAMP-bound RA (PDB: 1RGS) (15) were used. Automatic model building was achieved with Arp/wARP (26) and manual adjustment was made with Tufmod (27). Alternating cycles of model building and refinement, using the CNS program (22), were performed until the final R-factor and R-free dropped to 25% and 32% respectively. A PROCHECK analysis did not show any residue in the disallowed regions of the Ramachandran plot. Separately, we also set up crystallization of Rp-bound RA as follows. RA was purified and concentrated to 9 mg/ml using Vivaspin concentrators (Sartorius Stedim Biotech GmbH, Goettingen, Germany). A 2 mM aliquot of AMP-PNP was added and incubated for 3 days to allow for intrinsic ATP hydrolysis (24) and complete displacement of hydrolyzed ADP, to ensure saturation with the nonhydrolysable ATP analog (AMP-PNP). This was followed by overnight incubation with 1 mM Rp. Integrity of the holoenzyme was confirmed by analyzing the sample by size-exclusion chromatography (data not shown).

Crystallographic Data Collection, Structure Solution, and Refinement of Rp-Bound PKA RA

 purification of RA:C holoenzyme was concentrated to 9 mg/ml using Vivaspin concentrators (Sartorius Stedim Biotech GmbH, Gottingen, Germany). A 2 mM aliquot of AMP-PNP was added and incubated for 3 days to allow for intrinsic ATP hydrolysis and complete displacement of hydrolyzed ADP, to ensure saturation with the nonhydrolysable ATP analog (AMP-PNP). This was followed by overnight incubation with 1 mM Rp. Integrity of the holoenzyme was confirmed by analyzing the sample by size-exclusion chromatography (data not shown).

Crysallization, Data Collection, Structure Solution, and Refinement, of apo RA and cAMP-Bound RA—PKA RA was set up for crystallization at 25 °C in hanging drops using the vapor diffusion method in 0.1 M sodium cacodylate trihydrate pH 6.5, and 30% w/v glycerol using Spectra/pore 3.5 kDa molecular weight cut off membrane. The holoenzyme was further purified by size-exclusion chromatography (S75 column, AKTA FPLC system).

Crystallization, Data Collection, Structure Solution, and Refinement, of apo RA and cAMP-Bound RA—PKA RA was set up for crystallization at 25 °C in hanging drops using the vapor diffusion method in 0.1 M sodium cacodylate trihydrate pH 6.5, and 30% w/v polyethylene glycol 8000.

The crystals were transferred to a cryoprotectant solution (mother liquor containing 20% glycerol) and flash-frozen in liquid nitrogen. X-ray diffraction data were collected at the Beamline 9.1 (The Stanford Synchrotron Radiation Lightsource, CA). Diffraction data were processed and scaled using HKL2000. The final data were integrated and scaled in the space group P6_22 (a = b = 56.4, c = 168 Å) with satisfactory statistics shown in Table I. Initial phases of apo RA were generated by molecular replacement using the A-domain (residues 113–244) (PDB code 1RGS) (15) as a search model. Additionally, we also set up regions of the Ramachandran plot. Separately, we also set up crystallization of Rp-bound RA as follows. RA was purified and concentrated to 9 mg/ml using Vivaspin concentrators (Sartorius Stedim Biotech GmbH, Gottingen, Germany). A 2 mM aliquot of AMP-PNP was added and incubated for 3 days to allow for intrinsic ATP hydrolysis (24) and complete displacement of hydrolyzed ADP, to ensure saturation with the nonhydrolysable ATP analog (AMP-PNP). This was followed by overnight incubation with 1 mM Rp. Integrity of the holoenzyme was confirmed by analyzing the sample by size-exclusion chromatography (data not shown).

Crystallization, Data Collection, Structure Solution, and Refinement of Rp-Bound PKA RA—The purified RA:C holoenzyme was concentrated to 9 mg/ml using Vivaspin concentrators (Sartorius Stedim Biotech GmbH, Gottingen, Germany). A 2 mM aliquot of AMP-PNP was added and incubated for 3 days to allow for intrinsic ATP hydrolysis (24) and complete displacement of hydrolyzed ADP, to ensure saturation with the nonhydrolysable ATP analog (AMP-PNP). This was followed by overnight incubation with 1 mM Rp. Integrity of the holoenzyme was confirmed by analyzing the sample by size-exclusion chromatography (data not shown).

TABLE I

| Data Set | RA-Rp | apo RA | RA-cAMP |
|----------|-------|-------|---------|
| Space group | P3_21 | P6_22 | P3_12 |
| Cell constants (Å) | a = 66.9, c = 58.8 | a = 56.4, c = 168 | a = 62.64, c = 158.2 |
| Wavelength (Å) | 1.5418 | 1.00 | 1.00 |
| Average redundancy | 20.2 (15.1) | 4.4 (3.1) | 6.6 (6.7) |
| Completeness (%) | 99.9 (99.9) | 94.4 (95.0) | 99.4 (100) |
| R(reflect) = Σ(|I(h) - l(h)|) / Σ(l(h)), where l(h) is the mean intensity after rejection. | 30.55 (4.31) | 23.6 (10.3) | 16.8 (3.1) |
| Rfree = Σ(|I(h) - Fcalc(h)|) / Σ(Fcalc(h)); no l/α cutoff was used during refinement. | 5.7 (48.8) | |

Refinement

Resolution range (Å) | 50–2.3 | 50–2.7 | 22.3–1.5 |
| Number of reflection (Working/free) | 6648 (390) | 4160 (455) | 54558 (2772) |
| R.m.s. | 0.006 | 0.007 | 0.006 |
| Angles (°) | 1.2 | 1.4 | 1.317 |
| Rwork/Rfree (%) | 24/32 | 23.5/28.5 | 15/18 |

Crystal screens were performed at room temperature with Hampton and JB screens by the hanging drop vapor diffusion method. Reproducible crystals were obtained after 3 days in the JB 6 (D6) condition (100 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5), 2 M ammonium sulfate). Crystallization of PKA RA:C holoenzyme in the presence of 0.2 mM AMP-PNP and Rp generated crystals of RA-Apo bound to Rp alone. In both cases there was no trace of the C-subunit in the crystal. We again suspect the high salt in the crystallization conditions resulted in dissociation of the C-subunit. Prior to data collection, crystals were briefly soaked in a cryoprotectant consisting of paratone and mineral oil (1:1 ratio) and flash frozen in liquid nitrogen. A complete dataset was collected on a Bruker Platinum 135 CCD detector mounted on a Bruker Microstar H x-ray generator. The dataset was processed and scaled using the HKL2000 program (25). Matthew’s coefficient confirmed that the crystal was that of Rp-bound RA and not that of Rp-bound to the RA:C holoenzyme. The presence of R (and not cAMP or cGMP) was unequivocally confirmed, based on bromine density at the C8 position of Rp (Fig. 3). The structure was solved by molecular replacement, using the Molrep program (21) and the RA coordinates of the holoenzyme complex from the PDB file 3FHI (8) as the search model. Interestingly, the coordinates of the holoenzyme complex gave better solutions than when the coordinates of cAMP-bound RA (PDB: 1RGS) (15) were used. Automatic model building was achieved with Arp/wARP (26) and manual adjustment was made with Tufmod (27). Alternating cycles of model building and refinement, using the CNS program (22), were performed until the final R-factor and R-free dropped to 25% and 32% respectively. A PROCHECK analysis did not show any residue in the disallowed regions of the Ramachandran plot. Separately, we also set up crystallization of Rp-bound RA as follows. RA was purified and concentrated to 6 mg/ml. This protein was incubated with 1 mM Rp and screened for crystals. JB 5 condition (20% polyethylene glycol 8K, 100 mM 2-(cyclohexylamino)ethanesulfonic acid, pH 9.5) yielded crystals that we used for data collection, structure determination and refinement as described above.

Amide Hydrogen/Deuterium (H/D) Exchange Mass Spectrometry—

Amide Hydrogen/Deuterium (H/D) Exchange Mass Spectrometry—Apo RA was concentrated to 50 μM using Vivaspin concentrators (Sartorius Stedim Biotech GmbH, Gottingen, Germany). Samples were prepared by adding 1 mM cAMP, Rp and Sp each to 50 μM apo RA protein. 2 μl each of apo, cAMP-, Rp- and Sp-Ra in buffer (50 mM 2-(N-morpholino)ethanesulfonic acid, 200 mM NaCl, 2 mM EDTA, 1
structures of apo and cAMP-bound RA in the B-conformation and Rp-bound RA in the H-conformation. A, Structure of apo RA in the B-conformation showing the helical α-subdomain in brown and β-subdomain in tan. B, Structure of the cAMP-bound RA also in the B conformation is completely superimposable with apo RA except for cAMP bound to the PBC pocket. C, Structure of Rp-bound RA in the H-conformation showing the helical α-subdomain in smudge green and β-subdomain in tan. Structures determined independently from crystals of Rp bound to RA:C homodimer showing the helical α-subdomain in brown and β-subdomain in tan.

Hydrogen bonding distances (Å) between the ligands Rp and cAMP bound RA

| Atom1  | Atom2          | Rp:Rp | cAMP:Rp |
|--------|----------------|-------|---------|
| S1P (O1P) | Arg209 NH₂/NH₁ | 3.0   | 3.2     |
| S1P (O1P) | Ala202 N       | 3.3   | 2.6     |
| O2P    | Ala210 N       | 2.7   | 2.9     |
| O2     | Gly199 N       | 2.6   | 2.7     |
| O2'    | Glu200 OE1/OE2 | 3.1   | 2.8     |
| ArgNH₁ | Gly199 O       | 2.8   | 3.5     |

mm dithiotreitol were diluted with 18 μl D₂O (99.9%) to yield a final concentration of 90% D₂O. Exchange was carried out at 20 °C for various time points (0.5, 1, 2, 5, and 10 min). The exchange reaction was quenched by adding 40 μl of prechilled 0.1% trifluoroacetic acid to get a final pH read of 2.5. A 50 μl aliquot of the quenched sample (~130 pmol protein sample) was then injected on to a chilled nanoUPLC sample manager (beta test version, Waters, Milford, MA) as previously described (28). The sample was washed through a 2.1 × 30 mm immobilized pepsin column (Porozyme, ABI, Foster City, CA) using 100 μl/min 0.05% formic acid in water. Digested peptides were trapped on a 2.1 × 5 mm C18 trap (ACQUITY BEH C18 VanGuard precolumn, 1.7 μm resin, Waters, Milford, MA). The peptides were eluted using an 8%–40% gradient of acetonitrile in 0.1% formic acid at 40 μl/min, which was supplied by a nanoACQUITY Binary Solvent Manager onto a reverse phase column (Acquity UPLC BEH C18 column, 1.0 × 100 mm, 1.7 μm, Waters, Milford, MA). Peptides were detected and mass was measured on a Synapt HDMS mass spectrometer (Waters, Manchester, UK) acquiring in the MS² mode, a nonbiased, nonselective CID method (29–32).

Sequence identifications were made from MS² data from undeuterated samples using ProteinLynx Global Server 2.4 (beta test version) (Waters, Milford, MA) (32, 33) and searched against sequence of RA with no enzyme specified and no modifications of amino acids. Identifications were only considered if they appeared at least twice out of three replicate runs. The precursor ion mass tolerance was set at <10 ppm and fragment ion tolerance was set at <20 ppm. Only those peptides that satisfied the above criteria through Database search pass 1 were selected and are listed in Table III (32). The default criteria for false positive identification (Value = 4) was applied. A decoy database containing the reverse sequence of RA was created. No peptide identifications were found to be assigned to the decoy sequence and that of the C-subunit of PKA when the above default parameter for false positive identifications was applied. Increasing this parameter increased the number of identifications seen for the decoy sequence underscoring the stringency of sequence identifications with the default parameter setting. It should be noted that MS² does not produce quadrupole isolated tandem MS (MS/MS) spectra, and hence they are not optimal for submission to traditional search engines. Therefore to further confirm the identity of the pepsin fragments, we also carried out independent data dependent analysis of an undeuterated sample of RA. The MS/MS files generated were converted to .pkf files and searched against the RA sequence along with the decoy sequence used above with the Mascot search program (v2.1.0). A total of 14 peptide identifications corresponding to MOWSE scores ≥20 are listed in Supplemental Table I. This threshold MOWSE score was chosen because ions with scores of 12 and below also appeared in the decoy sequence. These results showed that MS² data searched with PLGS 2.4 maximized identification of peptides and were used for deuterium exchange analysis. These identifications were mapped to subsequent deuteration experiments using prototype custom software (HDX browser, Waters, Milford). Data on each individual peptide at all time points were extracted using this software, and exported to HX-Express (34) for analysis. A total number of 37 peptide fragments yielded primary sequence coverage of 93%.

Continuous instrument calibration was carried out with Glu-fibrinogen peptide at 100 fmol/μl. We also visually analyzed the data to ensure only well-resolved peptide isotopic envelopes were subjected to quantitative analysis. The lowest signal to noise ratio among all analyzed peptides was four. A control experiment was carried out to calculate the deuterium back exchange loss during the experiment by incubating ligand-free RA with deuterated buffer A for 24 h at room temperature. All reported deuterium exchange values were corrected...
cAMP Antagonist Promotes Selection and Preferentially Binds Inactive of Protein Kinase A

Molecular & Cellular Proteomics 10.3

10.1074/mcp.M110.004390–6

RESULTS

So far, structures of several end-point conformations of Rβ have been solved by x-ray crystallography. These include structures of cAMP-bound Rβ (15), C-subunit-bound Rα (8), and C-subunit-bound Rβ (13). These structures have provided molecular insights into the cAMP binding pocket and R-C interface, as well as provided details of the distinct B and H-conformational states. However a structural basis for Rp functioning as an inverse agonist and mechanistic details of cAMP-dependent activation of PKA, specifically by mediating conversion of the holoenzyme (H-form) to the cAMP-bound (B-form) conformation are still unclear. Also unclear is how apo Rα binds both cAMP as well as the C-subunit with equally high affinity. We believe that the apo as well as Rp-bound states represent critical intermediates in the interconversion of B and H-forms and mapping the structure and dynamics of these two states is critical to understanding the overall mechanism of cAMP action. Therefore we set out to solve the structures of the apo and cAMP-bound R-subunit fragment Rβ as well as Rp-bound Rα-C.

Structures of apo and cAMP-Bound Rα—In our 2.7 Å structure, a single apo Rα molecule occupies the asymmetric unit and only residues 108 to 243 could be traced, indicating high disorder in the N-terminal 17 residues spanning the pseudo-substrate site, as observed in the x-ray structure of cAMP-bound Rβ (15) (PDB: 1RGS). Furthermore, the overall topology of the molecule is identical to the cAMP-bound conformation (B-form), indicating that the apo form (Fig. 2A) is completely superimposable with the cAMP-bound form. However the structure of apo Rα is highly dynamic as it is characterized by high B-factor values for the main chain atoms throughout the molecule (Supplemental Fig. 2A).

The structure of cAMP-bound Rα was solved using molecular replacement with cAMP-bound Rβ (PDB: 1RGS) as the input model and this structure is highly similar to cAMP-bound Rβ (15) (Fig. 2B). The asymmetric unit of this 1.5 Å resolution structure contains two molecules. Interestingly, absence of the aromatic capping residue Trp 260, which is missing in this deletion mutant, is compensated by stacking interactions mediated by Tyr 188 from an adjacent molecule in the asymmetric unit.

Structure of Rα Bound to Rp—Although the two superimposable structures of apo and cAMP-bound Rα show differences in temperature factors (Supplemental Figs. 2A and 2B), there is no clear explanation to address the 1000-fold difference in affinity between the cAMP-bound and apo R-subunit for the C-subunit (20). Based on our recent report that Rp binding to Rα leads to a higher affinity for the C-subunit (19) wherein it functions more as an inverse agonist, we next set out to solve the structure of the PKA holoenzyme bound to Rp, which would represent an intermediate in cAMP-mediated activation. Despite using a nonhydrolyzable ATP analog (8), the obtained crystals were those of Rp-bound Rα alone. Dissociation of the C-subunit presumably could have resulted from the high ionic strength in the crystallization buffer (38). Rp-bound Rα crystallized in the trigonal space group P3_21. The structure was solved by molecular replacement using the conformation of the Rα in the holoenzyme complex (8) (PDB: 3FHI). The identity of the Rp ligand was confirmed by electron density for bromine at the C8 position. Rp binds in the syn conformation (Fig. 3) and the structure of Rp-bound Rα is identical to the C-subunit bound conformation (H-form) but not to the B-form (Supplemental Figs. 1 and 2). This was entirely unexpected and indicated that the basis for Rp functioning as an inverse agonist for PKA was because of its inability to induce a conformational change from the H to the B form (Fig. 2C). To test whether Rp functions as an inverse...
agonist only upon binding to the holoenzyme (preformed H-conformation), we crystallized apo Rsa bound to Rp in the absence of C-subunit. The structure of Rp-bound Rsa solved using these crystals was identical to that obtained with crystals of Rp-bound Rsa generated from co-crystallization of Rsa:C and Rp. These revealed that Rp-bound Rsa crystallized in the H-conformation (with Rp in the binding pocket) regardless of whether the C-subunit was absent (apo form) or present (holoenzyme; H-form).

Structural Differences Between Rp-Bound Rsa and apo, cAMP- and C-Subunit-Bound States—Superposition of the structures of Rp-bound Rsa with that of Rsa:C (8) shows a lower RMSD (0.910 Å) (Supplemental Fig. 1A) compared with the superposition with cAMP-bound Rsa (2.858 Å) (Supplemental Fig. 1B). This reflects that the Rp-bound Rsa assumes the H-form. More details are summarized below.

### β-Subdomain
- Rp is anchored within the PBC at the center of the β-barrel, by a similar network of contacts found in cAMP-bound Rsa (Table II, Fig. 3). Distances between the sulfur of Rp and critical residues (Ala 202 and Arg 209) of the PBC pocket are different from those seen for the equatorial oxygen of cAMP (Fig. 4). A particularly large increase in distance (~ 0.7 Å) is seen between the sulfur of Rp and the backbone amide nitrogen of Ala 202, a critical residue along with Arg 209 responsible for anchoring the cyclic phosphate of cAMP (15, 39). In contrast, the distances between the guanidinium moiety of Arg 209 and backbone carbonyl oxygen of Gly 199 are shorter, reflecting a stronger bond among these atoms in the Rp-bound structure.

Although, overall topologies of the PBC in the Rp-free and bound states are the same, a comparison of the communication networks in these structures reveal important differences.
These are primarily seen in the PBC and β-strands 2 and 3. Residues 161–163, which are part of the β-subunit, are disordered in the structure of Rp-bound RA. This reflects C-subunit-induced conformational changes in the R-subunit. In addition to the above residues, the carbonyl group of the highly conserved Gly 169, which is critical for the cAMP-mediated activation of PKA (40), makes a H-bond with Arg 226 in both the RA:Rp and Ra:C structures.

α-Subdomain—The conformationally mobile region of the α subdomain, the α:B/C helix region that forms part of the C-subunit-binding surface, adopts the H-conformation in RA:Rp structure.

Amide Hydrogen/Deuterium (H/D) Exchange Mass Spectrometry Analysis—To further probe conformational dynamics of the R-subunit, we carried out amide H/D exchange mass spectrometry experiments of apo, cAMP-, Rp-, Sp- and C-subunit bound RA. Earlier studies on the H/D exchange of the apo, cAMP-bound and holoenzyme states had shown clear evidence of allostery between the two subdomains of the protein (11). We were interested in extending this analysis to cover the two thio-substituted analogs. We set out to use electrospray ionization-quadrupole time-of-flight MS to measure the amide exchange, which yielded primary sequence coverage of ~93%.

Our amide H/D exchange results indicate that apo Ra is a highly dynamic molecule that shows high rates of amide deuterium exchange across the entire molecule, which directly correlate to the B-factor values of the backbone atoms (Fig. 5, Supplemental Fig. 2). cAMP binding causes a large decrease in amide exchange, suggesting higher ordering of several residues both within the PBC as well as in peripheral regions of the molecule. A nearly identical effect was also observed for Sp-bound Ra (Fig. 5, Table III). Although Rp binding did cause greater ordering of the molecule relative to the apo protein, there were significant differences in exchange at multiple regions, compared with the cAMP/Sp-bound proteins (Fig. 5). Most importantly, at the PBC, decreased exchange in the cAMP and Sp-bound states and intermediate exchange between the apo and cAMP-bound states was observed upon Rp binding (Fig. 5F). Within the PBC, the apo Ra and Ra:C showed highest amide deuteron exchange, consistent with the absence of ligand whereas cAMP and Sp-bound proteins showed lowest exchange. Interestingly, peptides within this region in Rp-bound Ra showed increased exchange, compared with other ligand bound proteins (Fig. 5F), reflecting potentially weaker interactions of Rp with Arg 209 and Ala 202. Subtractive analysis of the overlapping peptides showed that there were no significant differences at residues 203–206 as well as 213–217 between the Rp-bound and other ligand-bound states indicating that the most significant differences could be localized to peptides spanning residues Glu 200 and Ala 202 on one hand and Arg 209 and Ala 210 on the other. Our results also show that amide deuteron exchange at the N-terminal pseudosubstrate region (92–102) of Ra:C is lower than in the ligand-bound states (Fig. 5C), consistent with this region binding to the substrate binding cleft of the C-subunit. It is interesting to note that the highly disordered conformation of the pseudosubstrate region and N terminus appear to be independent of the B or H-conformational states of the cAMP-binding domain.

Crucial differences between the Rp-bound and cAMP/Sp-bound states are evident in the PBC. This is completely consistent with the differences in H-bonding distances seen in these residues from the crystal structures described in Table II. Subtractive analysis of peptides further localizes the differences to two regions within PBC that show differences. A first region is Glu 200 and Ala 202 (1.2 additional deuterons exchanged in residues 199–203 in Rp:Rα compared with apo).
pared with Sp/cAMP-bound RA) and a second region from 207–212 which includes important residues such as Arg 209 and Ala 210 that shows increased exchange of 1.6 deuterons in Rp:RA compared with the other ligand-bound states. Overall, Rp-bound RA shows significantly increased exchange across the entire PBC pocket reflecting a more dynamic occupancy of the PBC by Rp compared with Sp or cAMP.

**Ion Mobility Mass Spectrometry**—Although apo RA crystallized in the B-conformation, it still did not provide mechanistic insight into the basis for the 1000-fold difference in binding affinity for the C-subunit seen between the cAMP-bound and apo states of the protein. Amide H/D exchange did however reveal that the apo state was highly dynamic and distinct from the ligand-bound states. To further highlight

**Fig. 5.** Amide H/D exchange mass spectrometry shows that apo RA is highly dynamic and highlights clear differences in deuterium exchange in RA between Rp and other ligand-bound states. A, ESI-Q-TOF mass spectra for a peptide spanning residues RA(202–212) (m/z = 567.32(2)) comparing amide exchange in the apo, ligand-bound and C-subunit-bound states. (i) Undeuterated apo RA. (ii) The isotopic envelope for the same peptide from Sp-bound RA following 10 min deuteration (iii) The isotopic envelope for the same peptide from cAMP-bound RA following 10 min deuteration; (iv) The isotopic envelope for the same peptide from Rp-bound RA following 10 min deuteration; (v) The isotopic envelope for the same peptide from apo RA following 10 min deuteration. B, Time course of deuterium exchange at residues (202–212). Open circle (,), apo RA; Closed circle (●), cAMP-bound RA; Open diamond (♦), RAC complex; closed triangle (▲), Sp-bound RA; Closed Square (□), Rp-bound RA. C, Time course of deuterium exchange at residues 92–102. Symbols are as in Fig. 5B. D, Structure of apo RA highlighting regions showing enhanced deuterium exchange in the absence of ligands relative to CAMP-bound RA, in red. E, Structure of apo RA highlighting regions showing enhanced deuterium exchange in the Rp-bound state relative to Sp/cAMP-bound RA. F, Summary of deuterium exchange data for peptides spanning PBC in ligand-free and ligand-bound states. Overlapping nested peptides obtained greatly improve data resolution for the PBC region. The PBC region in Ra: Rp is more solvent accessible when compared with all other ligand bound states of the protein.
the differences in conformation between apo and ligand-bound states, we carried out ion mobility mass spectrometry studies to specifically probe differences in molecular shape between all the above states. Theoretical collisional cross-sectional area (CCS) measurements of the cAMP-bound (B-conformation) and C-subunit-bound RAB (H-conformation) from the structural coordinates of these two conformations predicted only small (~5% changes in CCS) changes that were below the resolution of our Synapt HDMS mass spectrometer. However, the changes predicted for the larger deletion construct encompassing both CNB-A and CNB-B, RAB, between the two conformations were much greater (~18%) and hence, we set out to determine experimental values for CCS for apo and ligand-bound states of RAB.

Three to four charge states (z = +9, +10, +11, and +12) were observed (Fig. 6A). A comparison of the drift times observed showed distinct differences between the Rp and Sp-bound states with the Sp-bound state showing a shorter drift time indicative of a more compact molecule compared with the Rp-bound state. This is entirely consistent with theoretical CCS measurements of the B and H-conformations with the H-conformation being larger (Fig. 6B). Interestingly, apo RAB shows a drift time distribution that spans both Sp and Rp-bound conformational states indicative of apo RAB existing as an ensemble of multiple conformations.

**DISCUSSION**

Conformational Selection in the R-Subunit: Rp Stabilizes Inactive H-Conformation—Conformational dynamics forms the molecular basis for the functioning of all signaling proteins. The ability of regulatory proteins to toggle between multiple conformations in response to ligand binding, other
proteins or post-translationally modified forms the basis for signal transduction. Recent experimental evidence has allowed postulation of a "conformational selection" hypothesis, which suggests that the ability of proteins to exist in ensembles of multiple conformational states is an inherent property of the protein (41). This introduces an additional dimension to earlier models that suggested binding events with extraneous proteins or ligands or post-translational modifications might be solely responsible for inducing conformational changes in proteins.

From our solution studies, we demonstrate that the ability of the apo form of the R-subunit to mediate mutually exclusive high affinity interactions with cAMP or C-subunit depends on its ability to exist in an ensemble of multiple conformations. This has been demonstrated clearly in the CNB from Epac by elegant NMR experiments where it has been estimated that the protein existed in an 80:20 distribution of B and H-forms (42). Although hints of such an ensemble were obtained from our crystallographic studies, amide exchange results show that the apo protein displayed the greatest amide deuterium exchange among all the states of the R-subunit tested. This suggested that the apo form of the R-subunit was conformationally highly dynamic and metastable. Our ion mobility mass spectrometry results show the apo R-subunit with a distinct profile for the collisional cross-sectional area that straddles the profiles of the cAMP-bound and Rp-bound states (Fig. 6C). This provides clear evidence that the apo R-subunit is indeed an ensemble of multiple conformational states and encompasses the two end-point B and H-conformations and is completely consistent with elegant NMR experiments comparing dynamics of Rp and Sp-bound Rα (43). Binding of cAMP or C-subunit would then occur through "conformational selection" where cAMP, which is a natural ligand, specifically binds to and selects the B-conformation whereas the C-subunit binds preferentially to the H-conformation. This clearly explains the 1000-fold difference in affinity between apo and cAMP-bound R-subunit for the C-subunit. Thus the higher affinity of apo R-subunit for the C-subunit is entirely because of "conformational selection" whereas C-subunit binding to cAMP-bound R-subunit (B-conformation) requires binding-induced dissociation of cAMP for stable holenzyme complex formation; "induced fit." This is supported by the observation that Rp functions more as an inverse agonist rather than a cAMP antagonist in the activation of PKA (19). It is interesting that a single thio substitution in Rp enables it to bind selectively to the H-form (Fig. 2C) in contrast to the cAMP-favored B-form (Fig. 2B). This indicates that the sulfur substitution at the equatorial oxygen is uniquely important for determining the preferred conformation that a ligand can bind. In summary, apo R-subunit is uniquely poised to interact with all ligands and the C-subunit via conformational selection whereas interconversion of stable end-point conformations as seen in the cAMP-dependent activation of PKA holenzyme is through induced fit (41).

Mechanism of cAMP Action and Basis for Antagonism of Rp—Although conformational selection describes the ability of Rp and cAMP to preferentially select the H-inactive and B-active conformations respectively, comparison of structures of the two states highlights the unique binding interactions of Rp relative to cAMP. Our structure of cAMP-bound Rα clearly shows the important contacts between the specific moieties of cAMP and residues within the PBC. These provide high specificity for cAMP binding to the conserved PBC pocket (2, 15). The important contacts include the anchoring of the 2′OH by both the side chain of Glu 200 and the backbone nitrogen of Gly 199. The axial oxygen of cAMP is anchored by a single contact with the backbone amide nitrogen of Ala 210. The adenine ribose mediates stacking interactions with Trp 188 from a neighboring molecule in the asymmetric unit while in solution, stacking interactions have been proven to be mediated by Tyr 244 (44). All the above interatomic distances are nearly identical between structures of cAMP-bound and Rp-bound Rα (Fig. 1 and Fig. 4). The importance of all these contacts for binding interactions has been confirmed by mutagenesis (7) and by the observation that 2′deoxy-cAMP is incapable of binding PKA R-subunit (45). These are listed in Table II and shown as black dashed lines in Fig. 4 and represent contacts important for binding and occupancy of PBC by cAMP and Rp. However, the distance between the equatorial sulfur and residues Ala 202 and Arg 209 are significantly different in Rp and is summarized in Table II and the H-bonds are represented by red dashed lines in Fig. 4. These confirm the importance of the equatorial oxygen not only for anchoring of cAMP to the pocket through critical interactions with the backbone nitrogen of Ala 202 and the guanidinium side chain of Arg 209, but also for allosteric communication through the above residues to activate PKA.

It is clear that the mechanism of cAMP action is through induced fit and involves binding to PBC followed by allosteric conformational changes. A combination of our x-ray crystallography and solution mass spectrometry results clearly indicates that whereas all moieties including the equatorial oxygen are important for binding, the equatorial oxygen is uniquely important for mediating allosteric relays necessary for propagation of the effects of cAMP binding. This is entirely consistent with this being the critical atom for determining whether preferred binding exists for the B or H-conformations. Details of the allosteric networks of residues leading from the equatorial oxygen are described below.

Comparison of our cAMP-bound and Rp-bound structures of Rα confirm the role of specific allosteric communication relays, proposed to propagate the effects of cAMP binding throughout the molecule to facilitate dissociation of the C-subunit. Amide exchange results further show very clearly that substitution of the equatorial oxygen by sulfur in Rp increases deuterium exchange within two specific regions of the PBC. These are the regions spanning Glu 200, Ala 202, and 207–212, spanning Arg 209. A network of conserved residues from
the PBC that have been demonstrated to be important for cAMP-mediated allostery of PKA are those A) between the hydrophobic core region of the PBC and the hinge region formed by the highly important α:B-C helices (46) and B) between Arg 209 of PBC with Asp 170 from strands β1-β2 (19); (40) (Fig. 4). The hydrophobic core of the PBC includes conserved residues, Leu 201, Ala 202, Leu 203 and Ile 204 responsible for communication with the α:B-C region via the Tyr 229 residue. This has been demonstrated in Epac2 by mutagenesis experiments to be an important conformational switch between the inactive H and active B-conformations (46) as well as NMR (47). The extensive conformational changes in this region in our structure of Rp-bound Rₐ and differences in amide exchange between cAMP-bound and Rp-bound states clearly validates the previous results.

Clear differences in the position of Asp 170, critical for a second relay via Arg 209, are seen between our structures of the cAMP-bound and Rp-bound Rₐ (Figs. 1 and 4). Previous mutagenesis (48) as well as structural comparison of the B (15) and H-conformational states (8) are consistent with our results.

Our findings confirm that the equatorial oxygen is critical for cAMP action and this is borne out by the important contacts that this atom mediates with residues of the PBC. This oxygen is positioned precisely to coordinate and bridge the interactions of the 2 OH of the ribose moiety together with the axial oxygen that are essential for binding, with the allosteric relays listed above. The basis for cAMP action is therefore a combination of stabilization of the PBC followed by allosteric relays propagated from the equatorial oxygen of the cyclic phosphate.

The S-P bond in phosphorothioates is more electronegative than the O-P bond of cAMP, making Rp a stronger acid relative to cAMP (49–51). This reduces the strength of H-bonding of the sulfur with both Ala 202 as well as Arg 209 (Table II, Fig. 4) leading to disruption of both allosteric relays. Substitution of the axial oxygen in Sp on the other hand has no effects on allostery. This is completely consistent from both amide exchange and x-ray crystallography, whereby increased deuterium exchange together with the increased interatomic distances seen in the crystal structure confirm that Rp binding to PBC is more dynamic than Sp/cAMP. Rp thus preferentially binds the inactive-H conformation through conformational selection while satisfying all the binding constraints associated with the “active-B” conformation.

Identification of Highly Selective Allosteric Inhibitors that Specifically Bind and Stabilize “Inactive” Conformations—Our results provide the following major insights into cAMP-dependent regulation of PKA. First, we demonstrate that the apo R-subunit is equally capable of binding cAMP or C-subunit through conformational selection although the dominant conformation is likely to be the H-form (42). This is because of the apo state existing as an ensemble of multiple conformations. This also explains its higher affinity for the C-subunit compared with cAMP-bound R-subunit. Second, the molecular basis for cAMP action is now better understood as occurring via binding interactions with PBC followed by specific allosteric conformational changes mediated solely by the equatorial oxygen of the cyclic phosphate. Thirdly, our results demonstrate the basis for antagonism of Rp. Substitution of the equatorial oxygen by sulfur alters the preferred conformation for binding to the H-form and disrupts all the allosteric conformational relays while retaining binding interactions necessary for docking to the PBC.

The above studies of the R-subunit provide insights into “rational” drug design. First it is important to obtain structural information of more than a single end point conformation of the target protein of interest. Complementary solution studies are equally essential to define the conformational ensemble behavior of the target in solution in the absence of effector proteins/ligands. Because the goal of inhibitor selection is to design ligands that maintain the target in an inactive conformation, it is necessary to distinguish between contacts on the protein necessary for binding from those important for inducing allosteric conformational changes. The ideal inhibitors are therefore those that satisfy all “binding” constraints without inducing conformational changes necessary for activation. This principle is evident from the synthesis of specific inhibitors such as Gleevec for Abl-kinase which specifically recognize and stabilize the inactive conformation and is a classic example of conformational selection. These classes of conformation-specific inhibitors show better target-specificity and are therefore more desirable (52). In our study, the key to how Rp functions as a mechanistic inhibitor of PKA, is that it too preferentially binds the inactive H conformation of the R-subunit as it contains all the important binding moieties yet it fails to induce allosteric conformational changes.

Our analyses also highlight and support increasing evidence emerging for the importance of protein flexibility (which allows conformational selection) in rational drug design. In addition to crystallographic snapshots, conformational dynamics of proteins in solution lead to better identification of small molecule disruptors of protein-protein interfaces, especially for proteins that tend to exist as conformational ensembles.

Acknowledgments—We thank Nilofer Husain, Balakrishnan S. Moorthy, Srinath Krishnamurthy and Wang Loo Chien for helpful discussions, Wang Xianhui, Lim Teck Kwang for mass spectrometry technical support and Yap Xiao Jun for assistance with protein purification.

* This work was supported by grants from A*STAR-Biomedical Research Council, Singapore to GSA and KS and a grant from Waters Corporation to GSA.

** To whom correspondence should be addressed: Department of Biological Sciences, National University of Singapore, Singapore 117543. Phone: +65 6516 7722; Fax: (65) 6779 2486; E-mail: dbgsa@nus.edu.sg or Department of Biological Sciences, National University.
CAMP Antagonist Promotes Selection and Preferentially Binds Inactive of Protein Kinase A

of Singapore, Singapore 117543, Phone: +65 6516 7932; Fax: +65 6779 2486; E-mail: dbks@nus.edu.sg.

ACCESSION NUMBER: Coordinates of apo-Rα, cAMP-Rα, and Rp-cAMPs-Rα were deposited in the Protein Data Bank (http://www.pdb.org) under accession codes 3IIA, 3PNA and 3PLQ, respectively.

REFERENCES
1. Rehnmann, H., Wittgenhofer, A., and Bos, J. L. (2007) Capturing cyclic nucleotides in action: snapshots from crystallographic studies. Nat. Rev. Mol. Cell Biol. 8, 63–73
2. Canaves, J. M., and Taylor, S. S. (2002) Classification and phylogenetic analysis of the cAMP-dependent protein kinase regulatory subunit family. J. Mol. Evol. 54, 17–29
3. Berman, H. M., Ten Eyck, L. F., Goodsell, D. S., Haste, N. M., Kornev, A., and Taylor, S. S. (2005) The cAMP binding domain: an ancient signaling module. Proc. Natl. Acad. Sci. U.S.A. 102, 45–50
4. Corbin, J. D., Cobb, C. E., Beebe, S. J., Granner, D. K., Koch, S. R., Gettys, T. W., Blackmore, P. F., Francis, S. H., and Wells, J. N. (1988) Mechanism of cAMP and cGMP-dependent protein kinases. Adv. Second Messenger Phosphoprotein Res. 21, 75–86
5. Amieux, P. S., and McKnight, G. S. (2002) The essential role of RI alpha in the maintenance of regulated PKA activity. Ann. N.Y. Acad. Sci. 968, 75–95
6. Poteet-Smith, C. E., Corbin, J. D., and Francis, S. H. (1997) The pseudo-substrate sequences alone are not sufficient for potent autoinhibition of cAMP- and cGMP-dependent protein kinases as determined by synthetic peptide analysis. Adv Second Messenger Phosphoprotein Res. 31, 219–235
7. Huang, L. J., and Taylor, S. S. (1998) Dissecting cAMP binding domain A in the R(II)alpha subunit of cAMP-dependent protein kinase. Distinct subsites for recognition of CAMP and the catalytic subunit. J. Biol. Chem. 273, 26739–26746
8. Kim, C., Xuong, N. H., and Taylor, S. S. (2005) Crystal structure of a complex between the catalytic and regulatory (R(II)alpha) subunits of PKA. Science. 307, 690–696
9. Cheng, X., Phelps, C., and Taylor, S. S. (2001) Differential binding of cAMP-dependent protein kinase regulatory subunit isoforms laipha and libeta to the catalytic subunit. J. Biol. Chem. 276, 4102–4108
10. Hamuro, Y., Anand, G. S., Kim, J. S., Juliano, C. S., Stranz, D. D., Taylor, S. S., and Woods, V. L., Jr. (2004) Mapping intersubunit interactions of the regulatory subunit (R(II)alpha) in the type I holoenzyme of protein kinase A by amide hydrogen/deuterium exchange mass spectrometry (DXMS). J. Mol. Biol. 340, 1185–1196
11. Anand, G. S., Hughes, C. A., Jones, J. M., Taylor, S. S., and Komives, E. A. (2002) Amide H2/D exchange reveals communication between the cAMP and catalytic subunit-binding sites in the R(II)alpha subunit of protein kinase A. J. Mol. Biol. 323, 377–386
12. Das, R., Esposito, V., Abu-Abed, M., Anand, G. S., Taylor, S. S., and Melacini, G. (2007) cAMP activation of PKA defines an ancient signaling mechanism. Proc. Natl. Acad. Sci. U.S.A. 104, 93–98
13. Kim, C., Cheng, C. Y., Saldanha, S. A., and Taylor, S. S. (2007) PKA-I holoenzyme structure reveals a mechanism for cAMP-dependent activation. Cell. 130, 1032–1043
14. Diller, T. C., Xuong, N. H., and Taylor, S. S. (2000) Type II beta regulatory subunit of cAMP-dependent protein kinase: purification strategies to accommodate between homologous binding sites (AI/AII and BI/BII) in cAMP kinase I and II. Biochemistry. 39, 8803–8812
15. Herberg, F. W., Bell, S. M., and Taylor, S. S. (2003) Expression of the catalytic subunit of cAMP-dependent protein kinase in Escherichia coli: multiple isoforms reflect different phosphorylation states. Protein Eng. 16, 771–777
16. Otwinowski, Z., and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 266, 307–326
17. Perrakis, A., Morris, R., and Lamzin, V. S. (1999) Automated protein model building combined with iterative structure refinement. Nat Struct Biol. 6, 458–463
18. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Improved methods for building protein models in electron density maps and the location of errors in these models. Acta Crystallogr A 47, 110–119
19. Anand, G. S., Krishnamurthy, S., Bishnoi, T., Kornev, A., Taylor, S. S., and Johnson, D. A. (2010) Cyclic AMP- and (Rp)-cAMPs-induced Conformational Changes in a Complex of the Catalytic and Regulator (R(α)) Subunits of Cyclic AMP-dependent Protein Kinase. Mol Cell Proteomics. 9, 2225–2237
20. Anand, G., Taylor, S. S., and Johnson, D. A. (2007) Cyclic-AMP and Pseudosubstrate Effects on Type-I A-Kinase Regulatory and Catalytic Subunit Binding Kinetics. Biochemistry. 46, 9283–9291
21. (1994) The CCP4 suite: programs for protein crystallography. Acta Crystallogr D Biol Crystallogr. 50, 760–763
22. Brüger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) CNS: Computer System and NMR system. A new software suite for macromolecular structure determination. Acta Crystallogr D Biol Crystallogr. 54, 905–921
23. Laskowski, R. A., Rullmann, J. A., MacArthur, M. W., Kaptein, R., and Thornton, J. M. (1996) AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. J Biomol NMR. 8, 477–486
24. Herberg, F. W., Bell, S. M., and Taylor, S. S. (1993) Expression of the catalytic subunit of cAMP-dependent protein kinase in Escherichia coli: multiple isoforms reflect different phosphorylation states. Protein Eng. 6, 771–777
25. Ruotolo, B. T., Benesch, J. L., Sandercock, A. M., Hyung, S. J., and Geromanos, S. J. (2009) Database searching and accounting of multiply phosphorylated precursor and product ion spectra from the data independent analysis of simple and complex peptide mixtures. Proteomics. 9, 1696–1719
26. Ruotolo, B. T., Giles, K., Campuzano, I., Sandercock, A. M., Bateman, R. H., and Langridge, J. I., Millar, A., and Vissers, J. P. (2002) A novel precursor ion discovery method on a hybrid quadrupole orthogonal acceleration time-of-flight (Q-TOF) mass spectrometer for studying protein phosphorylation. J Am Soc Mass Spectrom. 13, 792–803
27. Silva, J. C., Denny, R., Dorschel, C. A., Gorenstein, M., Kass, I. J., Li, G. Z., McKay, T., Nold, M. J., Richardson, K., Young, P., and Geromanos, S. (2000) Quantitative proteomic analysis by accurate mass retention time pairs. Anal Chem. 77, 6815–6820
28. Anand, G. S., Krishnamurthy, S., Bishnoi, T., Kornev, A., Taylor, S. S., and Johnson, D. A. (2010) Cyclic-AMP- and (Rp)-cAMPs-induced Conformational Changes in a Complex of the Catalytic and Regulator (R(α)) Subunits of Cyclic AMP-dependent Protein Kinase. Mol Cell Proteomics. 9, 2225–2237
29. Weis, D. S., Engen, J. R., and Kass, I. J. (2006) Semi-automated data processing of hydrogen exchange mass spectra using HX-Express. J Am Soc Mass Spectrom. 17, 1700–1703
30. Ruotolo, B. T., Giles, K., Campuzano, I., Sandercock, A. M., Bateman, R. H., and Robinson, C. V. (2005) Evidence for macromolecular protein rings in the absence of bulk water. Science. 310, 1658–1661
31. Ruotolo, B. T., Benesch, J. L., Sandercock, A. M., Hyung, S. J., and Robinson, C. V. (2008) Ion mobility-mass spectrometry analysis of large protein complexes. Nat Protoc. 3, 1139–1152
32. Mesle, M. F., Hunter, J. M., Schwartzburg, A. A., Schatz, G. C., and Jarroll, M. F. (1996) Structural information from ion mobility measurements: Effects of the long-range potential. J Phys Chem. 100, 10.1074/mcp.M110.004390-13
38. Corbin, J. D., Keely, S. L., and Park, C. R. (1975) The distribution and dissociation of cyclic adenosine 3′:5′-monophosphate-dependent protein kinases in adipose, cardiac, and other tissues. J. Biol. Chem. 250, 216–225
39. Canaves, J. M., Leon, D. A., and Taylor, S. S. (2000) Consequences of cAMP-binding site mutations on the structural stability of the type I regulatory subunit of cAMP-dependent protein kinase. Biochemistry. 39, 15022–15031
40. Abu-Abed, M., Das, R., Wang, L., and Melacini, G. (2007) Definition of an electrostatic relay switch critical for the cAMP-dependent activation of protein kinase A as revealed by the D170A mutant of Rlalpha. Proteins. 69, 112–124
41. Boehr, D. D., Nussinov, R., and Wright, P. E. (2009) The role of dynamic conformational ensembles in biomolecular recognition. Nat Chem Biol. 5, 789–796
42. Harper, S. M., Wienk, H., Wechselberger, R. W., Bos, J. L., Boelens, R., and Rehmann, H. (2008) Structural dynamics in the activation of Epac. J. Biol. Chem. 283, 6501–6508
43. Das, R., and Melacini, G. (2007) A model for agonism and antagonism in an ancient and ubiquitous cAMP-binding domain. J. Biol. Chem. 282, 581–593
44. Ringheim, G. E., Saraswat, L. D., Bubis, J., and Taylor, S. S. (1988) Deletion of cAMP-binding site B in the regulatory subunit of cAMP-dependent protein kinase alters the photoaffinity labeling of site A. J. Biol. Chem. 263, 18247–18252
45. Schwede, F., Maronde, E., Genieser, H., and Jastorff, B. (2000) Cyclic nucleotide analogs as biochemical tools and prospective drugs. Pharmacol Ther. 87, 199–226
46. Rehmann, H., Prakash, B., Wolf, E., Rueppel, A., de Rooij, J., Bos, J. L., and Wittinghofer, A. (2003) Structure and regulation of the cAMP-binding domains of Epac2. Nat Struct Biol. 10, 26–32
47. Das, R., Mazhab-Jafari, M. T., Chowdhury, S., SiDas, S., Selvaratnam, R., and Melacini, G. (2008) Entropy-driven cAMP-dependent allosteric control of inhibitory interactions in exchange proteins directly activated by cAMP. J. Biol. Chem. 283, 19691–19703
48. Gibson, R. M., Ji-Buechler, Y., and Taylor, S. S. (1997) Interaction of the regulatory and catalytic subunits of cAMP-dependent protein kinase. Electrostatic sites on the type I alpha regulatory subunit. J. Biol. Chem. 272, 16343–16350
49. Frey, P. A., and Sammons, R. D. (1985) Bond order and charge localization in nucleoside phosphorothioates. Science. 228, 541–545
50. Liang, C., and Allen, L. C. (1987) Sulfur does not form double bonds in phosphorothioate anions. J. Am. Chem. Soc. 109, 6449–6453
51. Basch, H., Krauss, M., and Stevens, W. J. (1991) Comparison of the electronic-structure of the P-O and P-S bonds. J. Mol. Structure-Theochem. 81, 277–291
52. Liu, Y., and Gray, N. S. (2006) Rational design of inhibitors that bind to inactive kinase conformations. Nat Chem Biol. 2, 358–364

In order to cite this article properly, please include all of the following information: Badireddy, S., Yunfeng, S., Ritchie, M., Akamine, P., Wu, J., Kim, C. W., Taylor, S. S., Qingsong, L., Swaminathan, K., and Anand, G. S. (2011) Cyclic AMP Analog Blocks Kinase Activation by Stabilizing Inactive Conformation: Conformational Selection Highlights a New Concept in Allosteric Inhibitor Design. Mol. Cell. Proteomics 10(3):M110.004390. DOI: 10.1074/mcp.M110.004390.