MAPPING cAMP SIGNALLING BY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

By

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ABSTRACT

Cyclic AMP (cAMP) is a second messenger that translates extracellular signals into tightly regulated biological responses. The cAMP binding domain (CBD) is a conserved regulatory switch that binds to cAMP and allosterically controls multiple cellular functions. All CBDs share a common architecture comprised of α- and β-subdomains. cAMP binds to the phosphate binding cassette (PBC) nested within the β-subdomain. In mammals the main cAMP receptors are protein kinase A (PKA), guanine exchange factors (EPAC) and ion channel proteins, including both the hyperpolarization-activated cyclic nucleotide-dependent channels (HCN channels) and the cyclic nucleotide-gated channels (CNG channels). Impaired activities of these proteins are associated with diabetes, cardiovascular diseases, cancer and Alzheimer’s disease. Therefore, these proteins represent promising therapeutical targets. However, the mechanism of their cAMP-dependent allosteric control is not completely understood. In the present thesis we have studied the allosteric mechanism of activation in PKA and EPAC using an NMR-based approach and we have proposed a model explaining how cAMP allosterically controls the activity of PKA and EPAC.

Binding of cAMP to the Regulatory (R) subunit of PKA facilitates the release of the Catalytic (C) subunit. According to our model, binding of cAMP triggers long range perturbations that propagate from the PBC to the R:C interface through both direct and indirect pathways. The indirect pathway involves two key relay sites located at the C-terminus of β2 (I163) and at the N-terminus of β1 (D170). D170 functions as an electrostatic switch that mediates the communication between the PBC and the helical subdomain, whereas I163 controls the global unfolding. Hence, removal of cAMP uncouples the α- and β- subdomains by breaking the circuitry of cooperative interactions radiating from the PBC. The proposed model was further validated by the cAMP agonist Sp-cAMPS and the cAMP antagonist Rp-cAMPS. It was observed that Rp-cAMPS, in which the equatorial exocyclic oxygen is replaced by sulphur, does not activate a necessary
indirect allosteric pathway, while its diastereoisomer (Sp-cAMPS) with opposite phosphorus chirality behaves similarly to cAMP activating all allosteric pathways. Our data also showed that the cAMP-antagonist stabilizes a ternary inhibitory complex between the effector ligand and both the regulatory and the catalytic subunits of PKA. At this point it is still not understood how the proposed model of cAMP allostery is conserved in other cAMP binding proteins such as EPAC.

EPAC is a multidomain guanine nucleotide exchange factor specific for small GTP-binding proteins and is directly activated by cAMP. We have probed how cAMP docks into the EPAC1 CBD and how its signal allosterically propagates from the cAMP binding site to the helical subdomain, which mediates the inhibitory interactions between the regulatory and catalytic regions of EPAC. Our comparative NMR investigation of cAMP signalling in PKA and EPAC revealed key functionally significant differences between these two systems that will facilitate the design of EPAC-selective therapeutics.
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LIST OF ABBREVIATIONS

AD: Alzheimer’s disease
APP: Amyloid Precursor Protein
BBR: Base Binding Region
C: Catalytic subunit of PKA
cAMP: Adenosine 3',5'-cyclic MonoPhosphate
cAMPS: Phosphorothioate analog of cAMP
CAP: Catabolite gene Activator Protein
CBD: cAMP-Binding Domain
CNG: Cyclic Nucleotide-Gated channels
CPMG: Carr–Purcell–Meiboom–Gill pulse sequence
CR: Catalytic Region
DEP: Disheveled-Egl-10-Pleckstrin
EPAC: Exchange Protein Directly Activated by cAMP
GEF: Guanine-nucleotide Exchange Factor
H/D: Hydrogen / Deuterium Exchange
H/H: Hydrogen / Hydrogen Exchange
HCN: Hyperpolarization-activated cyclic nucleotide-dependent channels
HSQC: Heteronuclear Single-Quantum Coherence 2D-NMR spectrum
IL: Ionic Latch
MS: Mass Spectrometry
NMR: Nuclear Magnetic Resonance
NOE: Nuclear Overhauser Effect
NTHB: N-Terminal Helical Bundle
PBC: Phosphate Binding Cassette
PF: Protection Factor
PKA: cAMP-dependent protein kinase A
ppm: parts per million
R(CBD-A): CBD A of R1α
R: Regulatory subunit of PKA
RA: Ras-Association domain
RC-CT-CPMG: Relaxation Compensated Constant Time CPMG pulse sequence
REM: Ras Exchange Motif
R1α: Isoform 1α of the R subunit of PKA
RMSD: Root Mean Square Deviation
RR: Regulatory Region
SASA: Solvent Accessible Surface Area
TM: Trans-Membrane
INTRODUCTION

CHAPTER 1
INTRODUCTION

1. Introduction

The significance of cAMP as a second messenger in cellular signaling was first reported by Sutherland in 1957. Since then, half a century has passed and the search for a cAMP dependent signaling mechanism is still continuing (1,2). It is now a dogma that the cellular signaling relies on the conversion of extracellular stimuli into tightly regulated intracellular responses. The signal transduction is initiated with the binding of hormones and effector molecules to the cell surface receptors called first messengers (Fig. 1). Cell surface receptors, such as the seven trans-membrane (7TM) helix receptors, are also known as G protein coupled receptors. These receptors can bind to a multitude of effector molecules. Based on the sequence similarities, 7TM receptors are classified into three families, namely A, B and C. Family A is the largest group of receptors which includes the receptor for light (rhodopsin), adrenaline (adrenergic receptors) and an olfactory receptor subgroup (3). Family B receptors are coupled to adenylyl cyclase through Guanine – nucleotide regulatory protein (G proteins) and have 25 members in the family including the gastrointestinal peptide hormone receptor family. The Ca$^{2+}$ sensing receptors, taste receptors and the GABA receptors are classified as family C.

The signal generated by the 7TM receptors upon binding to the effector molecule is subsequently transmitted inside the cell through the G protein coupled
to the receptor near the cell membrane. Martin Rodbell in 1971 proposed that the G-protein relays the signal from the 7TM receptor into the cell by stimulating adenylyl cyclase that generates the second messenger cAMP (Fig.1) (4). The G-protein is a heterotrimeric protein composed of three subunits (i.e. Ga, Gβ and Gγ). The Ga subunit is a Ras like guanine nucleotide binding entity that can interact with both GDP and GTP and has a GTP-hydrolyzing activity (5). G proteins are generally named after their α subunits; for instance the Gs protein is a heterotrimeric complex of Gαs coupled to adenylase cyclase stimulation. Likewise Gi proteins are associated with the inhibition of adenylase cyclase. In its inactive state, Gα binds to GDP and forms a heterotrimeric complex with one β and one γ subunit. Once an effector molecule binds to the 7TM receptor, the activated receptor forms a transient complex with the G protein. During this process, Gα will release the GDP to bind GTP and subsequently the protein dissociates from

FIGURE 1. Schematic representation of the cAMP signalling in eukaryotes.
the Gβγ complex. The activated GTP-bound Ga then activates the adenylate cyclase resulting in an at least locally elevated cAMP concentration in the cell. The intrinsic GTP-hydrolyzing activity of the Ga subunit assists the conversion of the active G protein back to the inactive heterotrimeric GDP-ligated state.

The endogenous level of cAMP is also controlled by phosphodiesterase (PDE), which selectively catalyses the hydrolysis of the 3'→5' phosphodiester bond of cAMP degrading it into AMP (2) (Fig. 1). In some cases PDEs are in turn up or down regulated by cAMP either directly or indirectly. For instance, binding of cAMP to GAF domains of PDE10A allosterically activates the protein, which is independent of cAMP binding to the catalytic subunit (6). In another example, the function of cAMP-specific PDE4D3 is up or down regulated by cAMP-dependent protein kinase A (PKA) mediated phosphorylation of either its regulatory or catalytic subunits (7). In addition to PDEs, another effective innate strategy to control the specificity and compartmentalization of cAMP signaling is the synchronized anchoring of cAMP binding domains to the A kinase anchoring proteins (mAKAP) at a specific location in the cell (2). For example, the muscle specific mAKAPs form a signaling complex with PKA, PDE4D3, ERK5 and EPAC1(8). Activation of PKA by cAMP activates PDE4D3 by phosphorylating the Ser54 residue of PDE that reduces the local concentration of cAMP. The mAKAP associated ERK5 on the other hand inhibits the PDE function by phosphorylation and the ERK5 activity in turn is attenuated by EPAC1, which is again recruited by PDE4D3.
1.1 \textbf{cAMP binding domain (CBD)}

The cAMP binding domains are found in both prokaryotes and eukaryotes and are coupled to a wide range of biological activities. For instance, in bacteria the cAMP binding domains are associated with the DNA binding proteins, while in mammals the binding of cyclic nucleotides is linked to protein phosphorylation, ion gated-channels, and other processes (9). In spite of the fact that cAMP-binding proteins mediate diverse functions, all cAMP binding domains (CBD) share similar characteristics (Fig. 2). CBDs allosterically control the functional subdomain of their respective coupled proteins and share a highly conserved eight-stranded beta sandwich topology forming a basket-like structure (Fig. 2c). The cAMP docks at the conserved phosphate binding cassettes (PBC) located at the centre of the basket (10-12). The PBC is comprised of a short helix and loop motif flanked by two beta strands (Fig. 2c). cAMP docks in such a way that the phosphate sugar group faces the PBC and forms a salt bridge with a highly conserved arginine at the C-terminus of the PBC.

Another structural feature common to all CBDs is the helix located at the C-terminal to the $\beta$-barrel. All known CBDs share this helix following $\beta8$, but they differ in its length. When cAMP binds to the PBC this helix is subject to a hinge-like rotation that brings it closer to the $\beta$-barrel region. This common helix is therefore commonly referred to as ‘hinge helix’. Following the hinge helix there is the poorly conserved lid region that often interacts with the base of the
Figure 2. General architecture of cyclic-AMP binding domains. a) Representation of the chemical structure of cAMP. b) The sequence alignment of the PBC from the cAMP binding domains of PKA I &II, EPAC2, CAP, HCN and CNG respectively. Residues highlighted in green are highly conserved, blue indicate residues conserved for functional groups and white indicates the least conserved residues. c) cAMP binding domain of Rla-8 (PDB 1RGS). All CBDs share common architecture for the β-barrel region (yellow), PBC, Hinge and lid. All 3D structures in this chapter are generated using the program pymol (13).

cAMP (11). Binding of cAMP to CBD triggers a conformational change that sends an allosteric signal from the PBC to the functional domain of the regulated protein. However, the allosteric mechanism of cAMP mediated signaling is not fully understood. Regardless of all the similarities among the known CBDs, there are some subtle differences among the CBDs of different cAMP regulated proteins that might play significant roles in cyclic nucleotide recognition. For example, in the PBC of cAMP dependent guanine nucleotide exchange factor (EPAC) the N-terminal Glu residue conserved in all other CBDs and forming a hydrogen bond with the 2' OH of the cAMP is replaced by either a Lys or a Gln (Fig. 2b). It has
been reported that mutations at this site in PKA completely abolish high affinity cAMP binding (10). It is therefore necessary to study all of the CBDs from different systems in great detail in order to devolve cAMP analogs as a specific drug targets.

1.1.1. \textit{cAMP binding domain in prokaryotes}

The catabolic gene activator protein (CAP) is a cAMP-binding protein in \textit{Escherichia coli} that regulates the expression of over 150 genes (14-16). cAMP binding to CAP induces an allosteric conformational change that leads to the binding of CAP to a specific DNA sequence near the RNA polymerase binding site. The structure of cAMP bound CAP was first solved by McKay in 1981 at 2.9 Å resolution, which was recently refined to 2.1 Å resolution (16). Historically, this is the first crystal structure of any cAMP binding domain. The CAP forms a homo-dimer, with each subunit folded into two domains: an N-terminal cAMP binding domain and a C-terminal DNA binding domain (Fig. 3). The CBD shows the usual fold with the antiparallel eight-strand β-roll structure. The PBC at the center of the β-subdomain forms a pocket where cyclic phosphate of the ribose ring docks (Fig. 3). In the CAP:cAMP crystal structure, two cAMP molecules bind per monomer. One cAMP binds to the PBC in \textit{anti} conformation (Fig. 3a), where the 2’ OH of ribose forms hydrogen bonds to E72 and G71, the equatorial
Figure 3. Ribbon diagram of catabolite gene activator protein bound to cAMP. The anti and syn conformation of cAMP is shown in the panel a and b, respectively. The anti or syn conformations are defined by the orientation of the nucleotide base of the cAMP with respect to the ribose ring. In anti conformation C₈ is positioned above the ribose moiety (a), whereas in syn conformation C₈ rotates to the opposite direction (b). The oxygen, nitrogen, carbon and phosphorus atoms are represented by red, blue, green and orange color respectively. In the panel c, CBD is indicated with darker shades of yellow, the PBC is represented by light blue color, the DNA binding domain is highlighted by green color. The cAMPS are represented by red sticks. Panel d illustrates the anti-cAMP binding at the PBC of cAMP binding domain. The hydrogen bonds are represented by dashed line, salt bridge is represented by double-sided arrow and the water molecules are shown by red balls. The structure is made from the PDB file 1C6N using the program pymol (13).
oxygen of cAMP forms a salt bridge to R82 and the adenine base interacts with T127 and S83 through water-mediated hydrogen bonds to N1 and N7 nitrogen atoms respectively (Fig. 3d) (16). A second cAMP molecule binds in syn conformation to the protein dimer interface at the DNA binding domain (Fig. 3b) (17). In the syn conformation, cAMP interacts directly with the protein through hydrogen bonds and hydrophobic interactions along with the water-mediated indirect interaction with DNA. The R180 residue of the main chain forms a hydrogen bond with the axial oxygen, which is also connected to the E181 through a water molecule. The detailed mechanism of the cAMP-mediated allostery is still not understood completely due the lack of high resolution biophysical evidence of the apo-state.

1.1.2. cAMP binding domain in eukaryotes

In mammals, one of the most important receptors for the cAMP secondary messenger is the ubiquitous and archetypical cAMP-dependent protein kinase A (PKA) (18). However, it is now established that cAMP also functions in a PKA-independent manner by binding and activating another major and recently discovered cAMP-receptor, the exchange protein directly activated by cAMP (EPAC) (19-21), which is a guanine-nucleotide-exchange factor (GEF) for the small GTPases Rap1 and Rap2. Together, PKA and EPAC mediate the majority of cAMP responses (20) resulting in the regulation of a multitude of diverse cellular processes that include ion-channel gating in the heart, insulin secretion in pancreatic β-cells, memory development, cell growth, cell adhesion, and cell
junction formation (Fig. 1) (10,22). The detailed structure and function of PKA and EPAC will be discussed later in this chapter.

Other cAMP-binding proteins found in eukaryotes are the hyperpolarization-activated cyclic nucleotide modulated channels (HCN) and the cyclic nucleotide-gated channels (CNG). The HCN family of proteins is involved in spontaneous rhythmic activity in both the heart and brain (23). Whereas the CNG family of proteins is found in photoreceptors, olfactory sensory neurons, and other neuronal and non-neuronal cell types (24). All of the cAMP-activated channel proteins have a C-terminal cAMP binding domain and a core transmembrane domain homologous to the voltage-gated K\(^+\) channels. The transmembrane domain exhibits a tetrameric cation channel architecture, where four \(\alpha\)-helical subunits form a pore that favors cation binding at the center of the membrane. Binding of cAMP to the CBD opens the channel completely and allows the passive movement of ions through the hydrophobic membrane core. The crystal structures of the CBDs for these proteins have been solved in both apo and holo states (25, 26).

The CBD of CNG has the characteristic fold of eight antiparallel \(\beta\)-strand rolls flanked by helices. The cAMP is docked in an \(anti\) conformation inside the PBC similar to the CAP protein, where R307 neutralizes the phosphate charge of the cAMP molecule and the 2' OH forms a hydrogen bond with the E298 of PBC (25). The \(\alpha\)C-helix interacts with cAMP through the side chain of R348, which forms a lid over the binding site. A similar cAMP capping has been reported for
Figure 4. cAMP-induced conformational rearrangement in CBD as illustrated from the overlap of cAMP-bound CNG structure (blue color, PDB 1VP6) and apo-CNG (orange color, PDB 1U12) structure, respectively. cAMP induces structural rearrangements in the hinge and lid regions whereas the β-subdomain remains unaffected. Removal of cAMP generates a wider binding pocket at the PBC, which is also coupled with the movement of the hinge and the lid away from the PBC. The horizontal dashed line shows the distance between A300 and P306 backbones in holo and apo structure, respectively.

the HCN channel, where R632 also interacts with cAMP through its side chains (26). The molecule exists as a dimer through the N-terminal helices. CNG is the only protein for which both the cAMP bound and free structures are known. In the absence of cAMP no significant structural rearrangement is observed for most of the β-roll region. In the PBC, E298 shifts away from the opposing residues in the hairpin loop, leaving a wider binding pocket (Fig. 4) (25). On the other hand, the α-helical region undergoes significant rearrangements upon the release of cAMP. The αC-helix no longer forms the lid over the PBC in the absence of cAMP and the αB-helix moves approximately 5 Å from the β-subdomain. The binding of cAMP also increases the total buried surface at the dimer interface. The proposed
model for the cAMP induced gating, based on the structure of CNG in apo and holo states, is that in the absence of cAMP the CNG channel is in the closed state. The structural rearrangement of the PBC and αC-helix induced upon cAMP binding moves residue L301 in the PBC and creates a cavity. Hence the cAMP-dependent rearrangement propagates from the PBC and αC-helix through the αA' and αA helices to the dimer interface, resulting in the opening of the gate (25).

1.2 Medical Relevance

1.2.1 Role of cAMP in Cardiovascular Physiology and Pathology

cAMP plays a fundamental role in the chain of reactions by which the β-adrenergic agonists affect the rate and force of the heart beat (27-29). For instance, the cAMP-mediated activation of PKA by epinephrine results in the phosphorylation of several substrate proteins, including troponin-I, which alters the cardiac relaxation, and phospholamban (PLB), which shortens the contraction period (27). In addition, incorrect cAMP signal translation by PKA has been linked to several vascular disorders such as acute ischemic stress in the brain and cardiac tumors (30, 31). For instance, the myxomas implicated in the Carney complex, a familial multiple neoplasia, are related to mutations in the R1α-subunit of PKA (30, 31). EPAC also plays a fundamental role in heart tissues (33). For example, in cardiomyocytes the concerted action of PKA and EPAC is essential as they coordinate two coupled cAMP-dependent feedback loops (20, 33).
1.2.2 **EPAC as a Target for the Treatment of Type II Diabetes and Alzheimer’s Disease.**

EPAC-selective cAMP-agonists have been proposed as promising drug leads for the therapy of hypoinsulinaemia and of Alzheimer’s disease (10). EPAC enhances insulin secretion from pancreatic β-cells in response to glucose, making this cAMP-receptor a potential target for the treatment of type II diabetes (10). In addition, two independent lines of evidence consistently indicate that EPAC is an excellent target for the treatment of Alzheimer's disease (AD) as well. First, EPAC activation stimulates the release of the soluble amyloid precursor protein (APP) ectodomain called sAPPα, which exerts potent neuroprotective and memory-enhancing effects (34). Second, EPAC expression levels are altered only in brain regions affected by AD (35, 36). These lines of evidence point to EPAC as a possible therapeutic target for AD. However, before effective AD drug candidates can be developed based on EPAC activation, it should be considered that PKA also plays a central role in the AD etiology. PKA is responsible for the pathogenic hyperphosphorylation of the tau protein, which is a hallmark of AD (37, 38). Hence, the cAMP-agonists with the highest therapeutic potential for AD must be selective for EPAC so that the release of the neuroprotective sAPPα is promoted, while the tau hyperphosphorylation by PKA is avoided.
In mammals, cellular functions are often regulated by phosphorylation and dephosphorylation of proteins. The family of enzymes that catalyse the phosphorylation of proteins are known as protein kinases. The protein kinase family constitutes 2% of the mammalian genome and 4% of the plant genome. Protein kinases are pivotal in the regulation of a multitude of cellular activities that include cell development, differentiation, responses to stress, metabolism, cell death, and memory (39). Impaired activities of these enzymes are associated with cardiac tumors, acute ischemic stress in the brain and obesity, which makes these enzymes excellent primary targets for therapeutic interventions (30, 40, 41).

In 1968, Krebs and co-workers purified PKA, which is responsible for the phosphorylation of enzymes involved in glucose metabolism, for the first time (42). cAMP-dependent protein kinase A, which is one of the simplest and best biochemically characterized enzyme to date (12), is considered to be a prototype for all other kinases (12, 43). PKA is ubiquitous in mammalian cells, where it exists in two main forms: the inactive tetrameric holoenzyme and the active free catalytic subunit (44) (Fig. 5). In the holoenzyme, two C molecules are non-covalently bound to the dimeric regulatory subunit (R). Upon binding to the cAMP second messenger, the R-subunit undergoes a conformational change that releases the C-subunit in its active state and makes it available for phosphorylation of downstream target proteins (45, 46). Conversely, the binding of the C-subunit
to the cAMP-bound R-subunit triggers the release of cAMP. The shuttling of the R-subunit between the cAMP-bound and C-bound conformations is therefore essential to control the enzymatic activity of the C-subunit.

\[
\begin{align*}
R_2C_2 + 4 \text{cAMP} & \xrightleftharpoons{\text{}} 2C + R_2(\text{cAMP})_4 \\
(\text{Inactive}) & \quad (\text{Active})
\end{align*}
\]

Figure 5. Overview of cAMP-mediated PKA activation. a) Equilibrium for the inhibitory control of the cAMP-dependent PKA. R and C denote the regulatory and catalytic subunits, respectively. (b) Schematic representation of the domain organization of R. The line connecting the Dimerization/Docking domain (DD) to the cAMP-binding domain A is the inhibitor linker region. Binding of cAMP to domain B cooperatively induces a second cAMP binding to domain A, which then allosterically controls the release of the catalytic subunit. (c) The open conformation of the catalytic subunit is represented (PDB 1J3H). The N-lobe (40-119) and the C-lobe (128-300) are shown in
shades of yellow and brown respectively. The Ser53 at the tip of the glycine rich loop, Phospho-Thr197, and Asp184 at the active site are highlighted by red balls.

1.3.1 The catalytic subunit of PKA

The catalytic subunit is conserved in all the members of the kinase super family. It is a 250 residue bi-lobal protein that includes a highly dynamic small N-lobe (amino terminal lobe) and a short linker region connecting a large carboxy terminal lobe called C-lobe (47). The small N-lobe is predominantly β stranded and binds to ATP, whereas the α helical C-lobe contains the catalytic machinery as well as the substrate docking site (Fig. 5c). In addition there are the N–terminal and the C-terminal tails anchored to the N- and C–lobes, respectively. The highly conserved C-tail is an integral part of the catalytic site. In contrast, the structurally unconserved N-tail is essential for the kinase localization. For instance, A kinase interacting protein (AKIPI) binds to the N-tail and facilitates its transport into the nucleus (48).

The mechanism of phosphoryl transfer by the catalytic subunit has been elucidated from the work of S. S.Taylor, R. Roskoski, and C. D. Ashby during last two decades (57, 49-51). It is now believed that ATP preferentially binds first to the enzyme, followed by the substrate peptide. Next, the γ-phosphate of the ATP is rapidly transferred to the peptide with a subsequent release of ADP. The cycling of the enzyme between the closed and open states through the intermediate conformation is essential for the activity of the enzyme (12). The
open conformation provides maximum access to ATP, which in-turn facilitates the peptide binding. On the other hand, the closed conformation brings the ATP and the acceptor peptide close together to ensure efficient transfer of the γ-phosphate of ATP (47). The N-lobe in the apo-enzyme (open conformation) is more dynamic compared to the C-lobe (52). In the binary complex (with either ATP or peptide) or the ternary complex (with ATP and peptide) the enzyme assumes a closed conformation with significant quenching of dynamics, as judged based on the temperature factors of the crystal structure (53-55).

1.3.2 The Regulatory Subunit of PKA

The R-subunit is a multi-functional, multi-domain and highly-dynamic protein. Even though there are four known R-subunit isoforms (RIα, RIβ, RIIα and RIIβ), they all share a common architecture (Fig.5b) comprised of an N-terminal dimerization/docking domain followed by a flexible linker region that includes the auto-inhibitory segment and is C-terminally connected to two tandem cAMP binding domains (12). The dimerization/docking domain mediates the subcellular localization of PKA by binding to the A Kinase Anchoring Proteins while interactions with the C-subunit are mediated by the auto-inhibitory region. The C-terminus of the linker region is connected to two homologous cAMP-binding domains (CBDs) A and B, respectively (12). The function of the CBD B is mainly to regulate the access of cAMP to domain A (56), the main central controlling unit that implements the interdependence between the cAMP- and the
C-binding sites in the regulatory subunit (Fig. 5b). Specifically, yeast two-hybrid screening and deletion mapping of regulatory subunit-1α (R1α) have revealed that the deletion mutant R1α (94-244) contains the primary components necessary for interaction with the C-subunit (43).

Figure 6. Ribbon diagram of regulatory subunit of PKA-I. a) The 3D structure of the R1α shows two distinct cAMP binding domains A and B, respectively (PBD 1RGS). The conserved β-barrel region is highlighted with yellow, Xn-A helix with orange, the PBC with blue, and the B/C helix in domain A and in domain B with red and purple, respectively. cAMPs in both of the domains are represented as red balls. b) Illustrates the cAMP docking at the phosphate binding domain of CBD-A. The conserved residues E200, G199, A202, R209 and A210 are indicated. The cAMP binding is stabilized through the hydrogen bonds with the protein, as shown by dashed lines, and stacking interactions with the aromatic ring of W260.
The crystal structure of the regulatory subunit bound to cAMP was solved by crystallizing the Δ1-91 deletion mutant of R1α (57) (Fig. 6a). The carboxy terminal of cAMP binding domain A is linked to domain B through a short linker region. The cAMP binding domain forms a ‘V’ shaped pocket that is conserved in all cyclic nucleotide binding proteins (57). The binding of cAMP to the PBC is stabilized through several hydrogen bonds as well as electrostatic and hydrophobic interactions (Fig. 6b) (57, 58). The nucleotide is sandwiched between β strands 6 and 7, the base binding region (comprised of β4-5) and the C helix. cAMP directly interacts with the Glu200 and Arg209 of the PBC in domain A and Glu324 and Arg333 in domain B, respectively. In contrast to CAP, the cAMP conformation in both domains of the R- subunit is syn. In addition to these electrostatic interactions, hydrophobic interactions originating from the Ala189 (β5), Val184 (β4), Ala210 and Ala211 (PBC) in domain A also contribute to the nanomolar binding affinity. Additionally, the stacking interaction between the indole ring of W260 in the lid region and the adenine base further stabilizes cAMP binding. Similar interactions are also present between the cAMP and domain B, except in this case the aromatic stacking is through Y371. The mutation of these residues in either of the two PBC domains of R1α reduces the binding affinity of cAMP (54, 58-60) but none of the mutations affect the catalytic subunit binding.
1.3.3 The R, C Interaction

Within the R-subunit there are two main sites that interact with the C-subunit. The first interaction site is the auto-inhibitory region that contributes a μM affinity for C by docking to its active site cleft (61) (Fig. 7). The second interaction site is contained within the α-subdomain of the cAMP-binding domain A (62) and together with site 1 leads to tight binding ($K_D \sim nM$) to C (43, 49). The details of the interaction between site 1 and the C-subunit have been elucidated by the structure of the ternary complex of the C-subunit, ATP, and the R-subunit (55, 63). The structure of the ternary complex between R:C:ATP reveals the mechanism of inhibition of the C-subunit by the R-subunit and explains how cAMP allosterically activates the holoenzyme.

In the holoenzyme complex the R-subunit undergoes a major structural reorientation upon cAMP release and the unstructured linker region docks at the active site of the C-subunit (Fig. 7 a-e). The C-subunit adopts a completely closed conformation where the docking of the inhibitor peptide nucleates the binding interface between the R- and C-subunits. Central to this interface is the hydrophobic interaction between Y247 in the catalytic subunit and Y205 in the PBC and I99 in the inhibitor site of RIα. This interaction has been hypothesized as the rate limiting step of the holoenzyme formation that facilitates the dislodging of cAMP by stretching the PBC (55). The global structure of the β-barrel region however remains largely intact in the ternary complex, whereas the helical subdomain undergoes complete reorganization (Fig. 7 b-d). The αB and αC helix
in the cAMP bound state forms a helix kink helix (lid region) and becomes a single continuous straight long helix in the R:C complex and interacts with the activation loop of the catalytic subunit (Fig. 7 b-c).

**Figure 7. R:C complex reveals the mechanism of PKA inhibition.** a) Crystal structure of the R(91-244):C complex of PKA (PBD 1U7E). In the R:C complex the catalytic subunit assumes a close conformation. The N-lobe and C-lobe of the catalytic subunit are colored according to Figure 5c. The non-hydrolysable analog of ATP, ANP is shown as balls. The regulatory subunit-A (119-244) is colored according to Figure 6 and the inhibitor linker region is highlighted in red. b) The detailed hydrophobic interaction nucleated from the inhibitory linker region, PBC and B/C helix is shown. The regulatory subunit is shown as cartoon and the residues are suffixed with R and the catalytic subunit is shown as a space-filling model where the residues are suffixed with C. c) The conformational rearrangement of the B/C helix in R1α subunit upon cAMP binding is shown. The
cAMP-bound structure is shown in orange and the R:C complex is coloured as described before. d) The dramatic reorientation of the Rα domains A and B upon R:C complex formation (PDB 2QCS). The regulatory subunit is shown as a ribbon model and the catalytic subunit is represented by van der Waals surfaces. e) The critical salt-bridge between R366-E261 necessary for the cross-talk between the two domains of the regulatory subunit is highlighted.

The cooperative mechanism of inhibition of the C subunit by domain B and domain A of R subunit was recently explained by the complex structure between the catalytic subunit and the full length regulatory subunit (R1αΔ91-379, R333K) of PKA (63). Upon binding to the C-subunit the two domains of R1α move around 60 Å and adopt an extended dumbbell shape (Fig. 7e). In the holoenzyme complex, the aromatic capping residues move away from the PBC. For example, the W260 that is at the N-terminal of the domain B stacks with the cAMP in the domain A, in the cAMP-bound state, and moves 30 Å to interact with the catalytic subunit in the holoenzyme complex (Fig. 7d). This residue actually forms the vital link between the two domains that talk through the conserved salt bridge between E261 and R366 (63) (Fig. 7e). In the cAMP-bound state, the bridging residues in the regulatory subunit are 15 Å apart from each other. Hence, it is hypothesized that the binding of cAMP to domain B will recruit the capping residue Y371, thereby breaking the salt bridge. The movement of W260 towards domain A will be favored and consequently stabilize the binding of cAMP (63). At this point, however, it is still not known how cAMP allosterically controls the release of the C-subunit form domain A.
1.4 **cAMP-dependent guanine nucleotide exchange factor (EPAC)**

Until recently it was thought that PKA was the sole receptor of cAMP in mammalian cells. However in 1998, two groups simultaneously reported a cAMP-dependent guanine nucleotide exchange factor for the Ras-like small GTPase Rap1 and Rap2, which is commonly referred to as EPAC (9, 19, 21). Since then, EPAC has been reported to play significant roles in the regulation of various cellular processes including insulin secretion, cell adhesion, cell polarity, and secretion (9). Two homologous variants of cAMP-binding human EPAC proteins are currently known, which are EPAC1 and EPAC2 (20). Both EPAC isoforms share a similar multi-domain organization with an N-terminal regulatory region (RR) and a C-terminal catalytic region (CR) (Fig. 8) (20). In the absence of cAMP, the RR inhibits the guanine-nucleotide-exchange activity of the CR; i.e. the cAMP-free (apo) state of EPAC is ‘auto-inhibited’ (Fig. 8a) (20, 64). Upon binding to the cAMP second messenger, the RR undergoes an as yet uncharacterized allosteric conformational change that attenuates the ‘auto-inhibition’ of EPAC, thus activating the guanine-nucleotide-exchange function of the CR (Fig. 8a) (20, 64).

### 1.4.1 The Regulatory and Catalytic Regions (RR and CR) of EPAC

In EPAC1 the RR contains a Disheveled-Egl-10-Pleckstrin (DEP) domain followed by a CBD (Fig. 8a) (20) While the DEP motif controls membrane localization, the CBD plays a key role in the regulation of EPAC (20). The
EPAC1 CBD recognizes cAMP and allosterically propagates the cAMP signal to the catalytic region. EPAC2, like EPAC1, contains the DEP-CBD motifs (Fig. 8a) (20), but only in EPAC2 the DEP domain is preceded by an additional CBD (Fig. 8a) that does not however alter the EPAC auto-inhibition or cAMP binding to the other CBD (20). Both EPAC1 and EPAC2 share a similar domain organization in the catalytic region (Fig. 8a) with an N-terminal Ras Exchange Motif (REM), which serves as a connection domain between the RR and the CR, and a C-terminal CDC25-homology domain (CDC25HD), which implements the guanine-nucleotide-exchange (GEF) function of EPAC (10). Between the REM and CDC25HD modules, EPAC2 contains a ubiquitin-like Ras-Association (RA) domain (Fig. 8a) (20).

1.4.2 RR-CR Interactions in EPAC

The structure of EPAC2 in its apo or ‘auto-inhibited’ state (Fig. 8 c, d) has been recently solved by X-ray crystallography (65), revealing a ‘closed’ overall conformation in which the regulatory region covers the CDC25HD domain and sterically hinders access of Rap proteins to the catalytic region (Fig. 8c). The apo EPAC2 structure has also unveiled that such inhibitory steric hindrance is secured by a group of salt bridges between the CDC25HD domain and the N-terminus of the second CBD. Such ionic interactions are spatially well clustered and are collectively referred to as the ‘ionic latch’ (Fig. 8) (65). Mutational studies suggest that cAMP-binding to the CBD N-terminal to the REM domain disrupts the ionic latch and causes an allosteric conformational change within this CBD, resulting in
a rigid body rotation of the whole regulatory region relative to the catalytic region (65).

Figure 8. Domain organization of cAMP dependent EPAC 1 & 2 are shown in panel a. The black filled circles symbolize functionally relevant cAMP ligands. The empty cAMP circle for the CBD:A of EPAC2 means that the domain is not required for the regulation of EPAC2. The function of the EPAC1 segment homologous to the EPAC2 RA domain is currently unknown and is therefore labeled with a question mark. DEP symbolizes the Disheveled-Egl-10-Pleckstrin domain, REM
stands for Ras-Exchange Motif, RA is the Ras-Association module, and CD25HD is the Homology Domain (CD25HD) Guanine-nucleotide-Exchange Factor (GEF) (b) Qualitative domain arrangement in EPAC2 upon cAMP binding. The apo-state is based on the recently reported ‘closed’ X-ray structure (65), while the cAMP-bound state model is only hypothetical and is based mainly on preliminary mutational data (20). In the apo-state, access of Rap to the CR is sterically hindered by the RR. After cAMP binding, such hindrance is removed by a cAMP-driven allosteric conformational change of CBD:B. Rap can then bind the CDC25HD domain and the guanine-nucleotide-exchange is activated. Panels c and d show the ribbon diagram of full length EPAC2 and the CBD (PBD 2EYV), the individual domains are colored according to the scheme in panel a.

The rotational motion in turn opens the access of Rap proteins to the catalytic domain (Fig. 8). While this model is still currently only hypothetical, it clearly points out that it is the cAMP-triggered allosteric conformational change of the CBD N-terminal to the REM domain that activates EPAC. Hence this CBD represents the ‘central controlling unit’ of EPAC. Given the high degree of homology between EPAC 1 and 2 (69% for the common CBD) and considering that EPAC1 is simpler (i.e. single CBD), more ubiquitous and has been linked to AD (10) and to signaling in cardiomyocytes (33), we will therefore focus on the CBD of EPAC1.

1.4.3 The EPAC1 CBD

No structure is currently available for EPAC1, but preliminary homology modeling (66) based on the structure of apo EPAC2 shows that the apo EPAC1 CBD preserves the general secondary structure architecture typical of CBDs with a contiguous β-subdomain forming a well-defined eight-stranded jelly-roll β-barrel and a non-contiguous all α-helical subdomain (Fig. 8). The cAMP binding
site is embedded within the $\beta$-subdomain, whereas several sites that are known to be critical for the activation of EPAC based on prior mutational analyses are confined within the $\alpha$-subdomain (65, 67). These regions include the ionic latch in the N-terminal $\alpha$1 and $\alpha$2 helices (Fig. 8) as well as two other loci located in the $\alpha$6 helix ('hinge', Fig. 8) and in the region C-terminal to it ('lid', Fig. 8).

In summary, all the CBDs share a common architecture comprising of eight antiparallel $\beta$-strands flanked by $\alpha$ helices (Fig. 2c). The PBC is positioned in the core of the $\beta$-sandwich scaffold where cAMP docks. Due to a high degree of structural conservation, all CBDs have been predicted to undergo similar cAMP-dependent structural re-orientations in the PBC, the hinge, the lid and the NTHB. The binding of cAMP causes the movement of the hinge helix and the lid which then approach the PBC, in turn inducing a structural perturbation in the NTHB (9). In spite of all the similarities, CBDs occasionally differ in the mode of cAMP recognition. For example, unlike PKA where cAMP binds in a syn conformation (Fig. 2a), cAMP orients in an anti conformation when it docks to the CBD of CAP and CNG (9, 12) (Fig. 2b). The CBDs of PKA, CAP and EPAC also shows differential responses towards the modification of the nucleotide base of the second messenger. PKA cannot distinguish between cAMP and cGMP, whereas CAP and EPAC functions are inhibited by cGMP (16, 110). Therefore, to understand the cAMP signaling, here in this thesis I have presented a comparative study of CBDs of PKA and EPAC.
1.5 Objective:

The activation of the catalytic subunit of PKA depends on the conformational fluctuation of the regulatory subunit between the cAMP or the C bound states. While the structures of cAMP-bound and C-bound RIIα have been used to elucidate the mechanism of cAMP recognition and conformational rearrangements: for the holoenzyme formation, it is still not clear how cAMP binding to the R-subunit leads to the dissociation and activation of the holoenzyme. In other words, it is not known why the C-subunit preferentially interacts with the cAMP-free state of the R-subunit rather than the cAMP-bound form of the R-subunit. A major outstanding question is how the cAMP signal propagates from the PBC to the other functional regions critical for PKA activity. Urea denaturation experiments and time resolved fluorescence anisotropy measurements have shown a quenching of dynamics of the free R-subunit upon binding to cAMP (60, 68). Overall the cAMP-free state of the R-subunit appears to be more dynamic than the cAMP-bound state of the R-subunit and might involve an ensemble of conformations in equilibrium. It is therefore reasonable to hypothesize that the dynamics of the free R-subunit are relevant for the recognition and binding of the C-subunit. Another open question is the degree of co-operativity of the cAMP allostery in PKA and how well the cAMP-dependent allosteric signaling mechanism of PKA is conserved in other cAMP-binding proteins in mammals, such as EPAC.
EPAC proteins were discovered less than a decade ago and the EPAC structural biology is still in its infancy. While the recently solved structure of apo EPAC2 has unveiled the mechanism of EPAC inhibition, it is still not clear how cAMP removes that inhibition and activates the catalytic domain. Due to the lack of a structure and of biophysical characterizations at residue or atomic resolution for the cAMP-bound state of EPAC, the present knowledge about the mechanism of EPAC activation by cAMP is very limited. It is not clear how cAMP is recognized by EPAC or how such cAMP-recognition events are translated into long-range allosteric effects that lead to the activation of EPAC. Based on different responses of EPAC and PKA with point mutations and cAMP modifications (20, 69) it is hypothesized that both the recognition and the allosteric activation mechanisms of EPAC are different from those of PKA.

In order to address the currently unsolved problems and in order to experimentally verify the hypothesis proposed above, we will pursue several specific aims.

1.5.1 Develop an NMR methodology to study the sparsely populated cAMP-free R1α A domain.

The effect of cAMP binding can be mapped easily by comparing the cAMP-bound and cAMP-free states of the protein. However, for the R1α A domain, a direct free and bound comparison is challenging due to instability of the free-state. In Chapter 2, an NMR-based method has been presented to study
such a system. The problem was circumvented by using an equilibrium perturbation method in conjunction with NMR dispersion measurements.

1.5.2 Characterize at atomic resolution the cAMP-dependent allosteric network in PKA.

The binding of cAMP to the β-subdomain of the R-subunit causes distal changes extending to the α-subdomain where one of the C-subunit contact sites is located (55). The detailed characterization at atomic resolution of the structural perturbations of the R-subunit occurring upon cAMP binding represents an essential step for understanding the mechanisms that lead to the release and activation of the C-subunit. Considering that at least some of the states involved in cAMP-induced structural transitions are dynamic, NMR spectroscopy is a suitable tool to study those states (60, 68, 70-72). In Chapter 3, NMR chemical shift and H/D exchange mapping experiments were used to propose a three-shell model that explains how the cAMP signal propagates from the PBC to the distal C interaction sites.

1.5.3 cAMP-Dependent Allostery as the Basis for Agonism and Antagonism in CBDs.

The three-shell model proposed above was further refined in Chapter 4 by extending the NMR characterization to cAMP phosphorothioate agonists and antagonists (Sp- and Rp-cAMPS) and by investigating the functional dynamics of the R-subunit in several functional states. In this chapter, a model for
antagonism and agonism has been proposed based on the stability of the ternary complex between the C and R-subunit of PKA and its ligand (cAMP or phosphorothioate cAMPS analogs).

1.5.4 Solve the solution structure of holo EPAC1

In Chapter 6, the solution structure of cAMP-bound EPAC1 has been presented. The comparison of the apo and holo structures of EPAC explained how cAMP binding removes the inhibition of EPAC. The mechanism proposed for EPAC illustrates a general signaling pathway for the CBD, yet preserves a distinct signature for EPAC signaling. Figure 9 shows the schematic presentation of the proposed aims and the biophysical techniques used to achieve those aims.
1.6 **Biomolecular NMR spectroscopy**

The word *allostery* originated from the Greek word *allos* and the 19th century English word *stereo*, which means *other-space*. The classical biochemical definition of allostery is *the action in part of a protein in response to ligand binding at a site distal from the active site*. In other words, allosteric processes involve conformational rearrangement due to ligand binding or post-
translational modifications, like phosphorylation, that propagate the signal to the distal active site (72, 73). Often allosteric processes are coupled to dynamics where a molecule changes its coordinate as a function of time (73). In biological systems, protein dynamics span a wide range of time scales and often involves exchanges between ground and excited states which are sparsely populated but crucial for enzymatic activity, protein–protein and ligand recognition. A thorough understanding of structure and dynamics is therefore essential to comprehend protein function.

Solution Nuclear Magnetic Resonance (NMR) spectroscopy has the unique ability to study both structure and dynamics at atomic resolution (70). A combination of improvements made in recombinant protein expression and advancements in computational capabilities coupled to NMR technology have significantly boosted the capacity of solution structure determination in the last two decades. Target proteins for NMR studies are typically uniformly or selectively enriched with spin-active and stable isotopes such as $^{15}$N, $^{13}$C and $^2$H. Using advanced double or triple resonance NMR experiments the frequency of each spin active nucleus is assigned to a specific atom within the protein. Based on this NMR assignment it is then possible to obtain information about protein structure and dynamics. Figure 10 summarizes the different biological events in protein chemistry and their relative motions. In general these motions can be divided into three categories. The first is picoseconds to nanosecond (ps-ns) motion, which involves individual atom fluctuations or local motions of loops in
a protein. The second is microsecond to millisecond motions, which relates to most of the essential biological activities such as ligand binding, catalysis, and allosteric regulation. The third category includes slower motions involving breathing motion and protein folding and unfolding (70, 73).

**Figure 10. Outline of protein functional dynamics and NMR techniques.** Proteins experience a wide range of motions ranging from ps to days. Over time, NMR has evolved as a unique technique to study both structure and dynamics at atomic resolution.

### 1.6.1 Picosecond (ps) – nanosecond (ns) dynamics

The ps–ns dynamics of proteins can be probed by studying the relaxation rates of the backbone or side chain spins. Relaxation is a process by which the spins regain their equilibrium position after the application of an RF pulse (74). Application of an RF field initially perturbs the Boltzmann distribution, but the spins will eventually return to their original equilibrium position by exchanging energy through the modulation by molecular motions of anisotropic interactions, such as dipole-dipole interactions and chemical shift anisotropy (CSA) (75-77). In other words, relaxation rates will carry information on the overall tumbling of
the protein and its internal motions. Ps-ns motion information can be obtained from dipole-dipole interactions and CSA mechanisms while the μs-ms motions are obtained from the modulation of isotropic chemical shifts caused by chemical exchange. The \( ^{15}N \) relaxation rates for an NH spin pair system subjected to dipole-dipole and CSA interactions are obtained from \( R_1 \), \( R_2 \) and the heteronuclear cross relaxation rates (78) through the following equations:

\[
R_1 = (c^2/4)(J(\omega_H-\omega_N) + 3J(\omega_N)+ 6J(\omega_H+\omega_N)) + c^2 J(\omega_N)
\]

\[
R_2 = (d^2/8)[4J(0)+J(\omega_H-\omega_N) + 3J(\omega_N)+ 6J(\omega_H)+ 6J(\omega_H-\omega_N)]
\]

\[
+ (c^2/6)[4J(0) + 3J(\omega_N)] + R_{ex}
\]

\[
\sigma_{NH} = (d^2/4)[6J(\omega_H+\omega_N) - J(\omega_H-\omega_N)]
\]

Where, \( R_1 \) is the spin-lattice or longitudinal relaxation rate

\( R_2 \) is the spin-spin or transverse relaxation rate

\( \sigma_{NH} \) is the dipole-dipole cross-relaxation rate

\[ d = (\mu_0 J_{YN} \gamma_H / 8\pi^2) < r_{NH}^{-3} > , \quad c = \omega_N \Delta\sigma / \sqrt{3}, \quad \mu_0 \text{ is permeability of free space, } h \text{ is Planck's constant, } \gamma_N \text{ and } \gamma_H \text{ are the gyromagnetic ratio of } ^{15}N \text{ and } ^1H, \text{ respectively. } r_{NH} \text{ is the nitrogen-hydrogen bond length (1.02 Å), and } \Delta\sigma \text{ is the CSA of } ^{15}N \text{ spins in a polypeptide chain (-170 ppm).}

The transverse relaxation rates (\( R_2 \)) are also affected by chemical exchange (\( R_{ex} \)) in addition to the dipole-dipole interactions and CSA mechanisms (79).

The cross relaxation rates and the longitudinal relaxation rates combine to give the steady-state heteronuclear NOE:

\[
\text{NOE} = 1 + (d^2/4R_1)(\gamma_H/\gamma_N)[6J(\omega_H+\omega_N) - J(\omega_H-\omega_N)]
\]
Equations 1.1-4 show that the relaxation rates are defined as a linear combination of the spectral density function \([J(\omega)]\) sampled at 0, \((\omega_H), (\omega_N)\), \((\omega_H+\omega_N)\) and \((\omega_H-\omega_N)\) frequencies \((76, 80, 81)\). The \(J(0)\) and \(J(\omega_N)\) are referred as lower frequency spectral density values and \(J(\omega_H), J(\omega_H+\omega_N)\) and \(J(\omega_H-\omega_N)\) are higher frequency spectral density values. Assuming that motions are simply described as an isotropic diffusion of a rigid rotor, the \(J(\omega)\) is represented by the Lorentzian:

\[
J(\omega) = \frac{\tau_c}{1 + \omega^2 \tau_c^2}
\]

where \(\omega\) is the Larmor frequency and \(\tau_c\) is the correlation time. Hence, \(J(\omega)\) values provide information about motions in time-scales corresponding to the \(\omega\) frequency. In order to get all the five spectral density values, the three relaxation data sets \((R_1, R_2\) and NOE\) are analyzed with either the model-free or the reduced spectral density function approach \((76, 82-85)\).

1.6.2 Model-free analysis of relaxation data

The model-free analysis method was first proposed by Lipari & Szabo in 1982, and was further extended by Clore and coworkers in 1990. According to this method, all \(J(\omega)\) values and the internal correlation functions are modeled from the relaxation data through the use of a simple analytical function that depends on \(\epsilon\) limited number of model-free parameters. The model-free analysis is based on the assumption that the relaxation due to isotropic tumbling of a protein is independent from the relaxation due to internal dynamics \((22, 81)\). The
spectral density function for a system undergoing isotropic rotational diffusion is given by (79, 83):

$$J(\omega) = \frac{2}{5} \left[ \frac{s^2 \tau_m}{1 + (\omega \tau_m)^2} + \frac{(1-S_f^2)\tau'_f}{1 + (\omega \tau_f)^2} + \frac{(S_f^2-S_s^2)\tau'_s}{1 + (\omega \tau_s)^2} \right]$$  \hspace{1cm} 1.6

Where, $\tau'_f = \frac{\tau_f \tau_m}{\tau_f + \tau_m}$ and $\tau'_s = \frac{\tau_s \tau_m}{\tau_s + \tau_m}$

$\tau_m$ is the overall correlation time of the molecule. For an isotropic molecule

$$\tau_m = \frac{1}{\nu D_{iso}}$$  \hspace{1cm} 1.7

If the diffusion tension is anisotropic, equation 1.6 should be modified according to Palmer (2004) to include the different contributions of $D_\parallel$ and $D_\perp$ (76), where $D_\parallel$ and $D_\perp$ are the diffusion constants for an axially symmetric diffusion tensor. Therefore, it is essential to determine the diffusion tensor accurately in order to calculate the correlation time (86). $\tau_f$ is the correlation time for fast internal motions in the ps time scale ($\tau_f < 100\text{-}200\text{ps}$) and $\tau_s$ is the correlation time for slower motion where $\tau_f < \tau_s < \tau_m$. $S^2$, $S_f^2$ and $S_s^2$ are the generalized order parameters that characterize the amplitude of internal motion in the ps–ns time scale where, $S^2=S_f^2 S_s^2$. In other words, the $S^2$ value indicates the degree of spatial freedom of the N-H vector as a result of internal motion. If the value of $S^2$ is zero, there is no restriction on the internal motion (i.e. the N-H vector samples a whole sphere), whereas if $S^2$ equal to unity there are no internal motions (i.e. the N-H vector adopts a single well-defined orientation) (79, 83).

There are five different models for the spectral density functions that are used to analyze the relaxation data according to the model-free approach (79).
The five models consist of the following subsets of extended model-free parameters: M1) $S^2$; M2) $S^2, \tau_\ell, M3) R_{ex}; M4) S^2, \tau_\ell, R_{ex}; M5) \tau_\ell, S^2$ and $S_z^2$. Out of all of the models, M1 is the simplest model, while M3 and M4 are applicable to systems with high transverse relaxation rates because of contributions from the chemical exchange ($R_{ex}$) in the μs-ms range. In practice, models are selected on the basis of a non-linear minimization of a $\chi^2$ and an F-test, as previously described by Mandel and co-workers (1995) as well as by Auvergne & Gooley (2003) (79, 87).

1.6.3 Reduced spectral density analysis of relaxation data

Alternatively, the relaxation data could be globally analyzed through reduced spectral density mapping (85, 88). Equations 1.1, 1.2 and 1.3 show how the relevant spectral density values are computed at five different angular frequencies: 0, $\omega_N$, $\omega_H$, $\omega_N + \omega_H$ and $\omega_H - \omega_N$. In other words, the three relaxation rates $R_1$, $R_2$ and $\sigma$ are computed based on these five spectral density values and the equation for the relaxation rates can be expressed in following matrix form (88):

$$
\begin{bmatrix}
R_1 \\
R_2 \\
\sigma_{NH}
\end{bmatrix} =
\begin{bmatrix}
0 & 3A + cB & A & 0 & 6A \\
\frac{2}{3}(3\ell + B) & \frac{1}{2}(3A + B) & \frac{1}{2}A & 3A & 3A \\
0 & 0 & -A & 0 & 6A
\end{bmatrix}
\begin{bmatrix}
J(0) \\
J(\omega_N) \\
J(\omega_H)
\end{bmatrix}.
$$

where $A = \left(\frac{\mu_r}{4\pi}\right)^2 \frac{\gamma_N^2 \gamma_H^2 h^2}{4\gamma_{NH}^2}$ and $B = \frac{\Delta \sigma_N \sigma_H \gamma_N^2}{3}$ constitute the dipole-dipole and CSA contributions of the relaxation mechanism, respectively. In order to measure the
spectral density with only three experimental relaxation rates, it is assumed that
the spectral densities at high frequency are equal, i.e \( J(\omega_H) = J(\omega_H) - J(\omega_N) = J(\omega_H) + J(\omega_N) = J(<\omega_H>) \) (85, 88). The equation is then reduced to:

\[
\begin{bmatrix}
R_1 \\
R_2 \\
\sigma_{NH}
\end{bmatrix} =
\begin{bmatrix}
0 & \frac{3A + cB}{2} & \frac{7A}{13} \\
\frac{2}{3} (3A + B) & \frac{1}{2} (3A + B) & \frac{13A}{2} \\
0 & 0 & 5A
\end{bmatrix}
\begin{bmatrix}
J(0) \\
J(\omega_N) \\
J(<\omega_H>)
\end{bmatrix}
\]

Replacing the cross relaxation rates with steady-state heteronuclear NOE (89):

\[
\begin{bmatrix}
R_1 \\
R_2 \\
\gamma_N \text{NOE}^{-1} \\
\gamma_H \text{T}_1
\end{bmatrix} =
\begin{bmatrix}
0 & \frac{3A + cB}{2} & \frac{7A}{13} \\
\frac{2}{3} (3A + B) & \frac{1}{2} (3A + B) & \frac{13A}{2} \\
0 & 0 & 5A
\end{bmatrix}
\begin{bmatrix}
J(0) \\
J(\omega_N) \\
J(<\omega_H>)
\end{bmatrix}
\]

Where, \( T_1 = \frac{1}{R_1} \).

Therefore, the relaxation rates \( R_1, R_2 \) and NOE are sufficient to measure the
three values of the spectral density: \( J(0), J(\omega_N) \) and \( J(<\omega_H>) \). \( J(0) \) reports on
motions slower than the overall tumbling (\( \mu s - ms \)), \( J(\omega_N) \) on motions in the
intermediate time scale (slow ns), and \( J(<\omega_H>) \) accounts for the fast local
motions (ps-\( \mu s \)).

1.6.4 Microsecond(\( \mu s \)) to millisecond(ms) dynamics and chemical exchange

\( (R_{ex}) \)

Physiologically \( \mu s - ms \) timescale motions are most significant since the
majority of the biological events occur in this time scale (70). For instance,
enzymatic activity, allostery, and protein folding all occur in the \( \mu s - ms \) time
scale by modulating the dynamic equilibrium between different conformational
states of the protein. Thus, these processes will alter the magnetic environment
of the atoms in the protein by changing the chemical context or the surrounding atoms and consequently increasing the transverse relaxation rate \( R_2 \) by dephasing the transverse coherences (see equation 1.2). This phenomenon is commonly referred as chemical exchange \( (R_{ex}) \) (90).

Let's assume that a spin is involved in a two-site exchange process (70):

\[
A \xleftrightarrow[k_f]{k_f^{-1}} B
\]

1.11

Where \( k_f \) and \( k_f^{-1} \) are the forward and reverse rate constants, respectively. The chemical exchange rate constant \( k_{ex} \) and the exchange time constant \( \tau_{ex} \) is given by:

\[
k_{ex} = k_f + k_f^{-1} = \frac{1}{\tau_{ex}}
\]

1.12

If the Larmor frequencies for the spin at site A and B are \( \omega_A \) and \( \omega_B \), respectively, the chemical shift difference between the two sites is related to \( \Delta\omega = \omega_B - \omega_A \). Based on the relative magnitude of \( k_{ex} \) and \( \Delta\omega \), the chemical exchange kinetics are divided into three regimes: fast, intermediate, and slow. The fast exchange situation arises when \( k_{ex} > \Delta\omega \). In this case a single peak is observed at the population-averaged position. The intermediate exchange \( (k_{ex} \approx \Delta\omega) \) regime gives rise to broad peaks. The effect of slow exchange \( (k_{ex} < \Delta\omega) \) is just opposite to the fast exchange regime where two resolved broad peaks are generated representing the two exchanging sites (70, 74, 90). Therefore the resonance signal for the residue experiencing chemical exchange will have a
weaker signal and a broad line. Similar effects on $R_2$ could also arise due to amide-proton exchange with the solvent and anisotropic tumbling motion. Hence a specific set of NMR methods are required to probe without bias pure chemical exchange contributions to $R_2$.

The chemical exchange contribution to the transverse relaxation rate is given by (70):

$$R_{ex} = R_2 - R_2^0$$  \hspace{1cm} 1.13

Where $R_2^0$ is the transverse relaxation rate constant due to the dipole-dipole interactions and CSA mechanisms in the absence of chemical exchange contributions. The chemical exchange could be identified by lineshape analysis, Carr-Purcell-Meiboom-Gill (CPMG) or $R_{1p}$ $^{15}$N transverse relaxation experiments, as well as $^{15}$N heteronuclear ZZ-exchange experiments (90-95). Lineshape analysis is the original method used for identifying chemical exchange (50). This method is highly informative when used in conjunction with ligand or protein titrations. However, this method is not applicable if there is either inhomogeneous line broadening by nonspecific aggregation or in a system where titration is not feasible.

Chemical exchange in a wide range of time scales is quantified by measuring the transverse relaxation rates by pulse sequences in which the chemical shift evolution is suppressed such as the CPMG or the $R_{1p}$ technique (90-95). For an accurate identification of $R_{ex}$, as per equation 1.13, the value of
$R_2^0$ can be obtained by analyzing $R_1$, $R_2$, and NOE rates through the model-free formalism. In the $R_{1p}$ experiment, a strong $B_1$ spin-lock is applied during the transverse relaxation period, which contributes to the sample heating. Furthermore, this experiment is also limited by the inhomogeneity of the spin-lock field that might affect the accuracy of $R_2$ rates (96). Alternatively, information about the chemical exchange can also be ascertained from NMR dispersion measurements using a relaxation compensated constant time CPMG (RC-CT-CPMG) pulse sequence (94, 97, 98). The CPMG pulse train (i.e. $90_x \tau 180_y \tau n$) is based on the basic idea of spin echo proposed by Hahn in 1950 (74). Mittermaier and Kay (2006) (95) have explained the basic principle of the RC-CT-CPMG dispersion experiment where a variable number of refocusing pulses are applied during a fixed relaxation time interval. The decay rates $R_2^{eff}$ for a given CPMG strength ($\upsilon_{CPMG}$) are obtained from the following equation:

$$R_2^{eff} (\upsilon_{CPMG}) = \frac{-1}{T} \ln \frac{I(\upsilon_{CPMG})}{I_0}$$

Where $I(\upsilon_{CPMG})$ and $I_0$ are the intensities of a given cross-peak with and without the CPMG period, respectively, and $T$ is the duration of the CPMG pulse train. The residue specific rates are obtained from the fitting of $R_2^{eff}$ as a function of field strength ($\upsilon_{CPMG}$) to the two site exchange model (96). The exchange rates measured by the CPMG experiments can however be affected by off-resonance effects (96).
The chemical exchange between two states can also be identified by the ZZ exchange experiment, provided the two states are in the slow exchange in the chemical shift time-scale and both states are present at high enough levels to generate an observable signal (99-102). The longitudinal magnetization transfer between the two states is achieved either by two spin order (H\textsubscript{2}N\textsubscript{z}) or by \textsuperscript{15}N longitudinal magnetization transfer (N\textsubscript{z}). The latter process is much more efficient than the former due to slow auto-relaxation of N\textsubscript{z} when compared to that of H\textsubscript{2}N\textsubscript{z} (99, 101). Therefore, N\textsubscript{z} exchange experiments can be used to probe chemical exchange over a wider range of time scales. In addition to quantifying the exchange rates, this experiment can also be used to transfer assignment from one state to other (100, 102).

1.6.5 Slow millisecond to hour dynamics

In solution, a protein exists in a dynamic equilibrium between statistical ensembles of native states (103-105). In the native state, a protein is subject to local and more rarely global unfolding events. The local unfolding processes often occur over intermediate (\textmu s–second) time scales and are functionally important for the allosteric regulation of protein-ligand binding and enzymatic activity. The global unfolding processes typically occur in the slow (ms-hour’s) time scales (106). Amide-hydrogen exchange experiments have been extensively used to study the structural fluctuations of the native ensemble in
these time scales, since the methods based on CPMG are sensitive only to ~ms processes (104).

In the analysis of amide-hydrogen exchange rates, it is assumed that the protein undergoes conformational transitions between two states, commonly referred to as open and closed states (104, 107, 108). The two step NH exchange process is explained by the following equation:

\[
\begin{align*}
\text{(H)}_{\text{open}} & \xrightleftharpoons[k_{\text{op}}]{k_{\text{cl}}} \text{(H)}_{\text{closed}} \\
\text{(H)}_{\text{open}} & \rightarrow \text{(H)}_{\text{exchanged}}
\end{align*}
\]

In the open state, water protons readily exchange with the protein amide protons that are otherwise inaccessible to the solvent in the closed conformation. In the native state, the conformational fluctuation including global unfolding processes, results in an open state where the amide hydrogen atoms become exchange competent. The kinetics of the observed exchange rate \(k_{\text{ex}}\) is expressed as:

\[
k_{\text{ex}} = \frac{k_{\text{op}} \cdot k_{\text{ex,open}}}{k_{\text{op}} + k_{\text{cl}} + k_{\text{ex,open}}}
\]

Where \(k_{\text{op}}, k_{\text{cl}}\) and \(k_{\text{ex,open}}\) are the rate constants of opening, closing, and the rate of exchange in the open state, respectively. The rate of exchange in the open state depends on the amino acid sequence, pH and temperature. The mechanisms of exchange are classified in two regimes: EX1 and EX2 (104, 107, 108). If the rate of hydrogen exchange in the open state \((k_{\text{ex,open}})\) is greater than the \(k_{\text{cl}}\), the exchange follows the EX1 mechanism \((k_{\text{ex,open}} >> k_{\text{cl}})\). The exchange in this case is
mainly dominated by slowly exchanging amides through structural opening steps \((k_{ex} = k_{op})\). For the EX2 regime, the exchange in the open state is slower, making \(k_{el}\) faster than \(k_{ex,open}\) \((k_{ex,open} \ll k_{el})\). This is the most common amide-hydrogen exchange mechanism observed under pH 7. In practice, hydrogen exchange rates could be probed by hydrogen-deuterium (H/D) exchange measurements. The exchange rates are measured for the backbone amides by the intensity decay as a function of time after exposing the protein to D\(_2\)O solvent and using a series of 2D \(^1\)H–\(^{15}\)N heteronuclear single quantum coherence spectroscopy (HSQC) experiments to achieve atomic resolution. Amides that are fully exchanged with D\(_2\)O during the dead-time of this experiment \((i.e. \sim 20\) minutes) are not suitable for real-time monitoring. On the other hand, the residues that exchange fast with water \((i.e. \text{in the 100 ms range})\) are probed by hydrogen-hydrogen (H/H) exchange experiments \((104)\). The H/H exchange rates are effectively measured by the transfer of magnetization from water to protein NH groups using a CLEANEX-PM spin lock \((100)\). The advantage of implementing this scheme is that it removes the artifacts arising from intramolecular NOEs and from exchange relayed NOEs.
CHAPTER 2
Chapter Two Preference

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I conducted all of the experiments and data analysis described in this chapter.
Abstract

The understanding of allostery relies on the comparative analysis of macromolecules in their free and bound states. However the direct free vs. bound comparison is often challenging due to the instability of one of the two forms. This problem is effectively circumvented by using minor free/ bound equilibrium perturbations which are tolerated without compromising sample stability. The subtle equilibrium perturbations are still able to reveal significant apo/ holo differences if monitored by NMR experiments that are sensitive to minor populations within dynamic equilibria, such as NMR relaxation dispersion (NMRD) and hydrogen exchange (H/D and H/H) rates. These measurements are complementary to each other as they unmask how a ligand affects both the stable and the excited states of the free energy landscape for its protein receptor. The proposed equilibrium perturbation approach therefore significantly expands the scope of applicability of NMRD and hydrogen exchange experiments to the investigation of ligand-protein interactions in general, unveiling allosteric ‘hot spot’ maps for systems that have been traditionally elusive to direct free/ bound comparison.
2.1 Results and Discussions

Allostery is a fundamental mechanism for regulating biological function through ligand binding (1). While understanding the structural basis of allosteric control relies on the comparative analysis of macromolecules in their free and bound states, the direct free vs. bound comparison is often experimentally challenging due to the instability of one of the two forms (2). This is the case for the regulatory subunit (R) of the prototypical and ubiquitous protein kinase A (PKA), which is the main eukaryotic receptor for the cAMP second messenger (3). The cyclic-nucleotide binding (CNB) domain of R that interacts with and allosterically controls the PKA catalytic subunit (C) is poorly soluble in its free form and as a result only limited information is currently available at residue or atomic resolution for the apo state of this ancient CNB module (3), despite its relevance for both signaling and the cyclic nucleotide dependent allostery in general (3-6). Analogous experimental challenges frequently hinder the comparative analysis of both prokaryotic and eukaryotic macromolecules in the apo and holo states, because often one of the two forms is aggregation prone due to the exposure of hydrophobic patches upon ligand binding or release (2, 4, 5, 7).

Here we propose a general experimental strategy based on equilibrium perturbation NMR to effectively circumvent the drawbacks intrinsic to direct apo/ho/lo comparisons. The proposed method takes advantage of the ability of recently developed NMR relaxation dispersion (NMRD) (8,9) experiments as well as of hydrogen exchange (H/D and H/H) measurements (10,11) to probe (“spy”) with high sensitivity minimally
populated states within dynamic conformational equilibria. As a result, the experimentally challenging direct free/bound comparison can be replaced by another more easily implemented comparison, namely between one solution in which the protein is prepared in its most stable state (either free or bound depending on the specific system under investigation) and another solution in which the equilibrium is slightly perturbed by introducing a minor population of the least stable form of the protein. The reduced effective concentration of the unstable state and the continuous on/off ligand exchange ensure the stability of the second solution. In this sample the direct detection of the least stable form is ineffective due to its low population but it can still be indirectly investigated by NMRD and hydrogen exchange experiments.

Figure 1. Effect of the equilibrium perturbation on the backbone $^{15}$N relaxation dispersion ($\Delta R_2^{\text{eff}}$) measured for Rlα (119-244). $\Delta R_2^{\text{eff}}$ is the difference between the relaxation dispersion measured before and after the addition of 1 mM cAMP to 0.1 mM Rlα (119-244) dialyzed under non-denaturing conditions to remove excess unbound cAMP (Supplementary Material; Figures S2, S3). The $R_2^{\text{eff}}$ rates were measured at 309 K (50 mM MES pH 6.5, 100 mM NaCl and 0.02% NaN$_3$) and $\Delta R_2^{\text{eff}}$ was computed according to equations (S2-S5) (Supplementary Material) (8). All data were acquired at 700 MHz with a TCI cryo-probe. Additional experimental details are available in the Supplementary Material. The dashed lines below the graph indicate the 2° structure expected based on the coordinates for the cAMP-bound regulatory
subunit of PKA (3). The horizontal lines on the graph indicate the average $\Delta \Delta R_2^{\text{eff}}$ value ± the standard error. “PBC” denotes the cAMP binding site. The other gray regions indicate allosteric sites previously hypothesized based on mutatant and sequence analyses (13).

The effectiveness of the proposed equilibrium perturbation NMR approach is illustrated here through its application to the CNB domain that serves as the central controlling unit of the isoform Iα of PKA (RIα (119-244)) (3,12). RIα (119-244) includes a highly conserved structural module and it represents a good model for CNB domains in general (3-6,12,13). We therefore prepared two samples of RIα (119-244): in one the holo state was stabilized by the addition of a ten-fold excess of cAMP and in the other a minor (< 10-20 %) population of the apo form was created by removing excess free cAMP through dialysis under non-denaturing conditions (Supplementary Material). Both solutions were stable thus enabling NMRD measurements for both samples. Specifically, the $^{15}$N relaxation dispersion ($\Delta R_2^{\text{eff}}$) was measured using relaxation-compensated constant-time (RC-CT) CPMG experiments (8) at CPMG field strengths ($v_{\text{CPMG}}$) of 43 Hz and 472 Hz (Supplementary Material). Since the on/off ligand exchange occurs in the ~1 ms – ~100 ms timescale as indicated by the ROESY analysis (Figure S1), the difference in $\Delta R_2^{\text{eff}}$ ($\Delta \Delta R_2^{\text{eff}}$) between the two samples (Fig. 1) is a sensitive indicator of residues affected by nucleotide binding. This is confirmed by several higher than average $\Delta \Delta R_2^{\text{eff}}$ values observed in Figure 1 for residues 199-211, which comprise the known cAMP binding site of RIα (119-244) (phosphate binding cassette, PBC). Figure 1 also shows that the effect of cAMP binding propagates well beyond the PBC as expected for an extended nucleotide-dependent allosteric network of
interactions. The allosteric ‘hot-spot’ map revealed by the $\Delta \Delta R^2_{\text{eff}}$ measurement is fully consistent with existing independent hypotheses on the cAMP allostery based on site-directed mutagenesis as well as sequence analyses (5,13,14). For instance, the cluster of higher than average $\Delta \Delta R^2_{\text{eff}}$ values localized at the end of the $\beta_2$ strand (Fig. 1) confirms the hypothesis based on surface-matching (13) that identifies this region as a highly conserved secondary hydrophobic layer required for the stability of CNB domain-cAMP complexes (13). Similarly, the peak $\Delta \Delta R^2_{\text{eff}}$ values observed at the beginning of the $\beta_3$ strand (Fig. 1) are fully consistent with the observation that the D170A mutant is non-allosteric even though it preserves high binding affinity for both cAMP and the catalytic (C) subunit of PKA (14). This result highlights the importance of the D170-R209 interaction within the allosteric network that mediates the cross-talk between the cAMP and the C binding sites of R1$\alpha$. In addition, the higher than average $\Delta \Delta R^2_{\text{eff}}$ value measured for L233 located at the interface between the B- and C-helices confirms the previously proposed hypothesis on the cAMP-controlled repositioning of the kinase-interacting C-helix through hydrophobic hinge motions (3,5,13). Overall, the consistency between the results of Figure 1 and previous independent mutational and computational analyses corroborates the usefulness of the equilibrium perturbation NMRD approach for identifying residues involved in binding and allosteric networks.

Further insight about the nature of the conformational transitions controlled by ligand binding is obtained by extending the equilibrium perturbation method to other types of experiments that are sensitive to minor populations within dynamic equilibria.
These include hydrogen exchange (H/D and H/H) measurements that provide information complementary to the NMRD data (Fig. 1). Whereas the $\Delta R_{2}^{\text{eff}}$ enhancements of Figure 1 mainly reflect chemical shift variations between the apo and holo forms in their most stable states (8,9), the differences in the hydrogen exchange rates between the free and bound protein probe primarily how the ligand perturbs the more excited, partially

Figure 2. Effect of the equilibrium perturbation on H/D (a, b) and H/H (c, d) exchange rates for representative backbone amide protons of RI$\alpha_{(119-244)}$ (10,11). Open and filled circles respectively refer to data measured before and after addition of 1 mM cAMP to 0.1 mM RI$\alpha_{(119-244)}$ dialyzed under non-denaturing conditions to remove excess unbound cAMP (Supplementary Material). Other experimental conditions are as in Figure 1. Further details are available as Supplementary Material.
unfolded and exchange competent conformers within the free energy landscape. In order to fully evaluate the role of a ligand in remodeling the free energy landscape, it is therefore critical to assess its effect also on the hydrogen exchange rates. For this purpose the equilibrium perturbation approach is extremely useful as shown in Figure 2a,b, which illustrates how even minor variations in the relative apo/holo populations as those employed for the $\Delta\Delta R_2^{\text{eff}}$ measurements generate marked changes in the hydrogen exchange rates monitored in real time through H/D exchange experiments (10). The equilibrium perturbation method is not only instrumental in effectively circumventing the experimental problems associated with the direct determination by NMR of the exchange rates for the aggregation prone apo state of R1\(\alpha\) (119-244), but it also provides the additional advantage of increasing the number of detectable fast exchanging amide protons that in the pure apo state would have been fully exchanged within the dead time of the H/D experiment. Furthermore, if the hydrogen exchange is too rapid to be monitored even by equilibrium perturbation real-time H/D measurements, it can often be probed by H/H exchange pulse sequences such as the CLEANEX-PM (Fig. 2c,d) (11). Again, as shown in Figure 2c,d, the observation of the effect of ligand binding on the H/H exchange rates is critically enabled by the equilibrium perturbation strategy.

In summary, we have shown that the equilibrium perturbation NMR approach is an effective method to map ligand binding and allostery avoiding the problems frequently associated with the direct comparison of the ligand free vs. bound states, as often only one of the two states is stable and amenable to direct experimental characterization. Minor apo/holo equilibrium perturbations are tolerated without compromising sample stability.
and can still reveal relevant apo/holo differences if monitored through suitable NMR experiments such as NMRD as well as H/D and H/H exchange. Together these complementary measurements unveil how a ligand affects both the stable and the excited states within the free energy landscape of its protein receptors. We conclude that the equilibrium perturbation approach significantly expands the scope of applicability of NMRD and hydrogen exchange experiments to the investigation of ligand-protein interactions in general, unmasking allosteric maps for systems that have been traditionally elusive to direct free/bound comparisons.

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2.3 Foot Notes: Supporting information available that includes Materials and Methods details.

2.4 References


2.5 Supporting Material

2.5.1 Materials and Methods

2.5.1.1 Expression, Purification and NMR Sample Preparation of R1α (119 – 244)

The regulatory subunit (119-244) of PKA was sub-cloned into the pRSET expression system (Invitrogen). The protein was over expressed in *E. coli* BL21(DE3) strain and purified according to the protocol of Hamuro et al. 2004 (S1). In brief, the bacteria were grown in 2L of 15N enriched minimal media (Spectra 9, Spectra Stable Isotopes) for twenty hours at 22 °C after induction with 0.5 mM IPTG at an O.D.600 of 0.8. The cells were harvested, re-suspended in the lysis buffer (20 mM MES pH 6.5, 100 mM NaCl, 2 mM EGTA, 2 mM EDTA and 5 mM DTT) with protease inhibitors and lysed by
passing three times through a French press. Following the removal of cell debris by centrifugation at 20,000xg, the proteins in the clear lysate fraction were precipitated by slow addition of ammonium sulfate at 4°C to a final concentration of 45% (w/v). The precipitate was isolated by centrifugation at 20,000xg, re-solubilized in the lysis buffer and incubated overnight with a cAMP Sepharose resin at 4°C. After extensive washing of the resin with a high salt buffer (lysis buffer containing 700 mM NaCl), the protein was eluted with 25 mM, 35 mM and 40 mM cAMP (Sigma) solutions. Further purification was performed by gel filtration on a HiLoad 16/26 Superdex-S75 column (GE Healthcare) which was pre-equilibrated with 20 mM KH2PO4 pH 6.5, 100 mM NaCl and 1 mM cAMP. The eluted protein was dialyzed against 20 mM KH2PO4 pH 6.5, 100 mM NaCl for 36 hours with buffer changes every 12 hour and subsequently dialyzed against the buffer used for the NMR experiments (50 mM MES pH 6.5, 100 mM NaCl and 0.02% NaN3). The NMR sample was prepared by concentrating the protein to 0.1 mM and adding D2O to a final concentration of 5% (v/v). Prior to the NMR experiments, the NMR sample was passed through a 100 kDa ultrafiltration membrane (Millipore) to remove any potential high molecular weight oligomers. The sample prepared in this manner is here referred to as sample “A”. In order to ensure full quantitative saturation of the cAMP-binding site another solution (sample “B”) was prepared by adding cAMP to sample A to a final concentration of 1 mM. The main difference between samples A and
B is the excess of cAMP, which is present only in the latter solution as shown by the $^1$H 1D NMR spectra (Fig. S2). Sample A still contains significant amounts of cAMP-bound Rlα (119-244) as indicated by the signals of bound cAMP observed in Figure S2 as well as by the similarity between the HSQC spectra of samples A and B (Fig. S3). Both samples (A and B) with and without excess cAMP were stable and were used for the NMR dispersion and for the hydrogen exchange (H/H and H/D) measurements.

2.5.1.2 NMR Spectroscopy

All NMR spectra were recorded at a temperature of 309 K on a Bruker AV 700 spectrometer equipped with a TCI cryo-probe. In all experiments, the $^1$H and $^{15}$N carrier frequencies were set at the water resonance and at the centre of the amide $^{15}$N region, respectively. Unless otherwise specified, the $^{15}$N dimension was digitized with 128 complex points for a spectral width of 31.8 ppm and the $^1$H dimension was digitized with 512 complex points for a spectral width of 14.2 ppm. During $^1$H acquisition $^{15}$N was decoupled using the GARP pulse train implemented with an RF strength of 1.32 kHz. All pulse-field gradients had a sine-bell shape digitized with 100 points. All data sets were processed using Xwinnmr (Bruker Inc.) or NMRPipe (S2). Unless otherwise specified, a phase shifted squared sine bell window function was employed for both dimensions prior to zero filling. Unless otherwise specified, cross-peak intensities were measured using Sparky 3.111 (S3) with Gaussian line fitting for the determination of the fit heights and the error in
the fit heights was estimated by calculating the standard deviation for the distribution of the differences in intensities of identical peaks in duplicate spectra, as previously indicated (S4). $^1$H chemical shifts were referenced relative to DSS, while the $^{15}$N chemical shifts were indirectly calibrated using the corresponding $\gamma_N/\gamma_H$ ratio (S5). 

1D-Watergate: 1D spectra were acquired using the Watergate 3-9-19 spin-echo (S6) for water suppression. 8K complex points were used for a spectral width of 11.98 ppm. After 128 dummy scans, 64 transients were accumulated with an inter-scan delay of 1s. 

2D-ROESY: The 2D-ROESY spectra were acquired with spectral width of 11.98 ppm in both dimensions. The directly and indirectly detected dimensions were digitized with 1K and 200 complex points, respectively. An inter-scan delay of 1.2s was employed and 64 scans were accumulated per $t_1$ point. The mixing times were 15 ms, 30 ms and 40 ms long and the CW spin-lock strength was 2.5 kHz. All other experimental conditions are indicated in the caption of Figure S1. 

2D-HSQC: Unless otherwise specified, all the 2D-HSQC spectra acquired were sensitivity and gradient enhanced and included water-flip back pulses. Unless otherwise specified, after 128 dummy scans, 8 scans were accumulated per $t_1$ point using an inter-scan delay of 1 s. The backbone cross-peaks in the HSQC corresponding to the bound-state of RILα (119-244) were assigned using standard triple-resonance experiments as previously explained (S7).
2.5.1.3 Relaxation Dispersion NMR

The backbone amide $^{15}$N relaxation dispersions were measured using a constant-time relaxation-compensated (CT-RC) CPMG experiment (8) implemented with an inter-scan delay of 2.2 s and a total CPMG length ($T_{CP}$) of 93.3 ms. The $^{15}$N pulses used for the CPMG train had an RF strength of 3.1 kHz as tolerated by the Bruker 700 MHz TCI-cryoprobe. For the purpose of mapping allosteric networks two sets of CT-RC-CPMG experiments were acquired with CPMG field strengths ($v_{CPMG}$) (8) of 43 Hz and 472 Hz corresponding to eight and eighty-eight 180° $^{15}$N pulses, respectively. More 180° $^{15}$N pulses are not tolerated by the Bruker 700 MHz TCI-cryoprobe within the constant CPMG length. For each CPMG field strengths fifteen interleaved replicate data sets were collected each with 128 dummy scans and 16 scans per $t_1$ point. Data sets corresponding to the same $v_{CPMG}$ were co-added prior to processing. Peaks affected by partial or total overlap were excluded from the analysis of the CT-RC-CPMG spectra. No cross peak was observed for V134 and L139 due to their fast relaxation rates that weaken the signal beyond detection during the long relaxation delay ($T_{CP}$). The NMR relaxation dispersion ($\Delta R_2^{\text{eff}}$) was quantified as:

$$\Delta R_2^{\text{eff}} = R_2^{\text{eff}}(43 \text{ Hz}) - R_2^{\text{eff}}(472 \text{ Hz})$$  \hspace{1cm} (S2)

Considering that: $^8$  

$$R_2^{\text{eff}}(v_{CPMG}) = (-1/T_{CP})\ln(I_{v_{CPMG}}/I_0)$$  \hspace{1cm} (S3)
where \( I_{\text{CPMG}} \) and \( I_0 \) are the intensities of a given cross-peak with and without the CPMG periods, respectively, and where \( T_{\text{CP}} \) denotes the total CPMG length as indicated above, the relaxation dispersion was evaluated as:

\[
\Delta R_2^{\text{eff}} = \left( \frac{1}{T_{\text{CP}}} \right) \ln \left( \frac{I_{472\text{Hz}}}{I_{43\text{Hz}}} \right)
\]  

(\text{S4})

\( \Delta R_2^{\text{eff}} \) was measured both before (sample A) and after (sample B) addition of 1 mM excess cAMP to the sample dialyzed under non-denaturing conditions. The difference between the \( \Delta R_2^{\text{eff}} \) measurements for samples A and B is reported in Figure 1 as:

\[
\Delta \Delta R_2^{\text{eff}} = \Delta R_2^{\text{eff}}(\text{Sample A}) - \Delta R_2^{\text{eff}}(\text{Sample B}) \quad \text{(S5)}
\]

2.5.1.4 \textit{H/D Exchange}

The samples for the H/D exchange experiments were prepared by first concentrating the dialyzed protein (sample A) to 1 mM. Half of the sample (~250 \( \mu \)L.) was passed through a Sephadex G10 column with a 3 mL bed volume pre-equilibrated with 50 mM MES pH 6.5, 100 mM NaCl in 100% D\( _2 \)O at 4° C. After transferring the sample in the D\( _2 \)O buffer to the NMR tube, HSQC spectra were recorded with preset parameters for a sample with similar buffer and sample height in order to minimize the dead time of the H/D exchange experiment. The fast initial decay was monitored through a series of 30 HSQC spectra acquired with two scans (~10 minutes per HSQC). The remaining slower decay was monitored through a series of 44 HSQC spectra.
acquired with four scans. 1 mM cAMP was then added to the other half of the 1 mM protein sample and the buffer was exchanged to D$_2$O by passing the protein solution through the same column pre-equilibrated with the same buffer but with 1 mM cAMP added to it. The H/D exchange decays for this sample were measured as for the previous sample without excess unbound cAMP. The HSQC cross-peak heights were quantified by NMRPipe as the sum of the intensities in a 3 x 3 grid centered on the peak maximum (8). The error on the intensities was based on the standard deviation of the spectral noise (S2). The H/D decays in Figure 2a,b were normalized relative to the intensities in the 1$^{\text{st}}$ HSQC spectrum acquired after exposure to D$_2$O and fitted to mono-exponentials.

2.5.1.5 H/H Exchange

The H/H exchange rates between water and the backbone amide protons were measured using the CLEANEX-PM-FHSQC experiment (11) for both sample A (no excess unbound cAMP) and sample B (1 mM excess cAMP). The data were recorded with 32 scans per t$_1$ point and a 2 s recycle delay. The CLEANEX-PM mixing period was implemented with a RF strength of 6.9 kHz to minimize offset-effects. The CLEANEX-PM mixing time durations were 5 ms, 10 ms, 30 ms, 40 ms, 60 ms and 80 ms. For the short (5 ms and 10 ms) mixing times two replicate data sets were collected, which were then co-added. The reference peak intensities (I$_0$ in Figure 2c,d) were measured from the FHSQC spectrum used as detection block for the
CLEANEX-PM-FHSQC experiment (11). The CLEANEX-PM-FHSQC build up curves were normalized with respect to these reference FHSQC intensities.

2.5.2 References for Supplementary Material


Figure S1: Expansion from the 700 MHz 2D-ROESY spectrum ($\tau_{\text{mix}} = 15$ ms) acquired for 0.1 mM RI$\alpha$ (119-244) with 1 mM excess cAMP at 309 K in 100% D$_2$O, 50 mM MES pH 6.5, 100 mM NaCl and 0.02% NaN$_3$. The marked cross-peak is assigned to the adenine H2,8 aromatic protons. This cross-peak has the same sign as the diagonal peaks and arises from the cAMP exchange between the free and bound states. The intense signal from the free H2,8 cAMP protons caused $t_1$-noise along the indirectly detected dimension.
Figure S2: Expansions from the 700 MHz 1D-Watergate (S6) spectra acquired for 0.1 mM Ria (119-244) at 309 K (50 mM MES pH 6.5, 100 mM NaCl and 0.02% NaN3). The top trace was recorded in the presence of 1 mM excess cAMP (sample B), while the lower trace was recorded after dialysis under non-denaturing conditions and before addition of 1 mM cAMP (sample A). The labels show the assignment of selected protons from the adenine and the ribose of both free and bound cAMP. The intense signals from free cAMP are absent in the lower trace, while the signals arising from bound cAMP are not significantly perturbed.
Figure S3: Representative regions from the 700 MHz 2D-HSQC spectra acquired for 0.1 mM Rlα (119-244) at 309 K (50 mM MES pH 6.5, 100 mM NaCl and 0.02% NaN₃) in the presence and absence of 1 mM cAMP ligand excess. (a) Ten-fold cAMP excess (Sample B, as in the upper trace of Figure S2); (b) No cAMP excess (Sample A, as in the lower trace of Figure S2). The residue labels indicate cross-peak assignments. Further details are provided in the text of the Materials and Methods section.
cAMP ACTIVATION OF PKA DEFINES AN ANCIENT SIGNALING MECHANISM

CHAPTER 3
Chapter Three Preference

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I conducted all of the experiments and data analysis described in this chapter. Esposito, V. completed the resonance assignment of Rlα (119-244), Anand, G. contributed to new reagents and analytic tools.
CAMP ACTIVATION OF PKA DEFINES AN ANCIENT SIGNALING MECHANISM


Abstract

Cyclic AMP (cAMP) and the cAMP binding domain (CBD) constitute a ubiquitous regulatory switch that translates an extracellular signal into a biological response. The CBD contains α- and β-subdomains with cAMP binding to a phosphate binding cassette (PBC) in the β-sandwich. The major receptors for cAMP in mammalian cells are the regulatory (R) subunits of PKA where cAMP and the catalytic (C) subunit compete for the same CBD. The R-subunits inhibit kinase activity while cAMP releases that inhibition. Here we use NMR to map at residue resolution the cAMP-dependent interaction network of the CBD-A domain of R1α. Based on H/D, H/H and N$_2$ exchange data, we propose a molecular model for the allosteric regulation of PKA by cAMP. According to our model cAMP binding causes long-range perturbations that propagate well beyond the immediate surroundings of the PBC and involve two key relay sites located at the C-terminus of β$_2$ (I163) and at the N-terminus of β$_3$ (D170). The I163 site functions as one of the key triggers of global unfolding, while the D170 locus acts as an electrostatic switch that mediates the communication between the PBC and the B-helix. Therefore, removal of cAMP not only disrupts the cap for the B' helix within the PBC,
but also breaks the circuitry of cooperative interactions stemming from the PBC, thereby uncoupling the α- and β-subdomains. The proposed model defines a signaling mechanism, conserved in every genome, where allosteric binding of a small ligand disrupts a large protein-protein interface.

The cAMP and the cyclic nucleotide binding domain (CBD) are conserved from bacteria to man as a ubiquitous signalling mechanism to translate extra-cellular stress signals into appropriate biological responses (1). The major receptor for cAMP in higher eukaryotes, cAMP-dependent protein kinase (PKA) (2), is ubiquitous in mammalian cells where it exists in two forms: the inactive tetrameric holoenzyme and the active dissociated catalytic (C) subunit. In the inactive holoenzyme, two C subunits are bound to a dimeric regulatory (R) subunit (Fig. 1a). Upon binding cAMP, the R-subunits undergo a conformational change that unleashes the active C-subunits (3, 4). The R-subunits are composed of an N-terminal dimerization/docking domain, a flexible linker that includes an auto-inhibitory segment, and two tandem cAMP binding domains - CBD-A and CBD-B (Fig. 1b) (5). The CBD-A of RIα contains a non-contiguous α-subdomain, which mediates the interactions with the C-subunit and a contiguous β-subdomain which forms a β sandwich and contains the cAMP binding pocket (i.e. the phosphate binding cassette or PBC) (Fig. 1c,d) (6).

Crystal structures of CBD-A of RIα in its cAMP- (6) and C-bound (7) states have revealed two very different conformations highlighting the conformational plasticity of this ancient domain. Although these static crystal structures define two stable end points,
questions remain about the allosteric control of the reversible shuttling between the two states. How does the signal generated by cAMP binding to the PBC (Fig. 1c,d) propagate through a long-range allosteric network that spans both α- and β-subdomains? Previous analyses (8-16) have led to the proposal of an initial allosteric model in which the α- and β-subdomains are directly coupled to each other through a salt bridge between E200 and R241 and also possibly through a hydrophobic hinge defined by the L203, I204 and Y229 side chain cluster (9, 11, 12). However, mutations (17), sequence conservation analyses (1), structure based comparisons (1), and genetic screening (18, 19) indicate that several other sites, which are not accounted for by the existing model, are also likely to play an active role in the cAMP-mediated activation of PKA. To comprehensively understand this allosteric mechanism, it is therefore necessary to elucidate at high resolution how cAMP remolds the free energy landscape of CBD-A, which serves as the central controlling unit of PKA. For this purpose, we have investigated by NMR R1α(119-244), a construct that spans both α- and β-subdomains of CBD-A and retains high affinity binding to cAMP. Based on the Cα and Cβ ppm values this R1α segment preserves a very similar fold to that observed in a longer R fragment spanning both tandem CBDs (6) (Supplementary Material, Fig. S1), verifying that R1α(119-244) represents a good model for CBD-A.

Using a combined NMR approach based on H/D and H/H hydrogen exchange (Fig. 2) and chemical shift changes measured through N2-exchange spectroscopy (Fig. 3) we have mapped at residue-resolution the interaction networks that propagate the cAMP
signal within CBD-A. Our results define an allosteric model (Fig. 4) according to which cAMP-release breaks a long-range circuitry uncoupling the α- and β-subdomains and thereby unleashing distal “hot spots” that serve as primary interactions sites for recognition of the C-subunit. Our findings reported here represent the first successful attempt of studying CBD-A of the R-subunit by multidimensional solution NMR
methods. Furthermore, the proposed model defines a mechanism that is highly conserved and thus relevant for cAMP recognition in other homologous CBDs coupled to effector proteins with diverse functions, such as transcription factors (catabolite-activator protein, CAP) (20-31), guanine nucleotide exchange factors (EPAC) (11) and ion channel proteins (both hyperpolarization-activated cyclic nucleotide-dependent channels [HCN channels] and cyclic nucleotide-gated channels [CNG channels]) (32, 33). This model also serves as a general paradigm for how small molecules such as cAMP allosterically control large protein-protein interfaces.

3.1 Results and Discussion

3.1.1 cAMP-binding affects all the CBD-A sites directly or indirectly involved in C-recognition and causes increased solvent exposure.

The protection factors (PFs) based on the H/D and H/H exchange rates measured before (black symbols) and after (green symbols) excess cAMP was dialyzed out, are reported in Fig. 2a,b, respectively. The corresponding absolute and percentage differences in the logPFs are shown in Fig. 2d,e, respectively. The first key feature that emerges from Fig. 2a is the dramatic decrease in protection upon removal of excess cAMP for most of the residues within the PBC. Interestingly, a marked decrease in solvent exposure is observed not only for amide sites that are directly hydrogen bonded to cAMP, such as G199 and A210 (Fig. 2a and Table S1), but also for the other amides in the PBC. Indeed, when cAMP levels are sub-
stoichiometric, the entire PBC with the exception of L203, exchanges more rapidly with the bulk solvent. This behaviour is explained considering that removal of cAMP most notably removes the N-terminal capping mechanism for the PBC’s B’ helix (6), which is therefore destabilized. The B’ helix is one of the R:C interaction sites and it includes Y205, which nucleates a major hydrophobic interface between the R and C subunits (7). By releasing the helix cap generated through the cyclic phosphate, the removal of cAMP now frees up this tip of the PBC so that it is available to dock to the C-subunit. The PF variations within the PBC therefore provide an initial understanding of a first level of cAMP control for the R:C interactions.

An additional level of cAMP control for the R:C interactions is revealed by Fig. 2b which shows that cAMP removal results in increased solvent exposure also at several other R:C contact sites well outside the immediate PBC, consistently with the existence of a long-range interaction network nucleated by the docking of cAMP and impaired by the elimination of cAMP. For instance, the H/H-based PFs (Fig. 2b,e) clearly indicate that cAMP-release enhances the solvent exposure of both the XN/A-helix loop and the C-helix. These two motifs are both essential elements for docking R to C in the holoenzyme complex (7) and the cAMP-induced long-range perturbations at these sites are in agreement with the observed cAMP-dependent chemical shift variations (Fig. 3). These observations provide therefore an experimental proof of the importance of allosteric mechanisms that couple the PBC and the C-helix, such as the E200/R241 salt bridge (9) and the hydrophobic hinge (11, 12).
Figure 2b, e also reveals that other significant cAMP-induced changes in solvent shielding occur at sites not directly involved in R:C interactions. For instance, the maps of both the H/H-based PF variations (Fig. 2e) and of the chemical shift changes (Fig. 3b) point to a major cAMP-effect on the locus centred at D170 and located at the N-terminus of β3. This region is not involved in direct interactions with the catalytic subunit of PKA, however at this site significant local conformational changes are observed upon C-subunit binding both at the level of backbone local RMSD (Fig. S2) and of side chain orientation, with the D170 $\chi_1$ dihedral angle changing from 48° in the cAMP-bound to -107° in the C-bound states of CBD-A (Fig. 4e,f) (6, 7). This correlation between C-binding and the conformation of D170 suggests that the D170 region may serve as an additional site available for cAMP to control C-recognition by R. Another region of CBD-A which is not directly involved in C-binding but for which the local conformation is indirectly affected by C is the C-terminus of the $\chi_N$-helix (Fig. S2). Similarly to D170, the H/H-based PF maps (Fig. 2b,e) point to a decrease in solvent shielding upon cAMP-release also at this site. Therefore this locus may also offer further opportunities for controlling the R:C recognition by cAMP.

3.1.2 The H/D exchange of the β-subdomain inner core amides is concerted by transient global unfolding events and it is highly cAMP-sensitive.

Another remarkable feature of the PF variations reported in Fig. 2d is that the most dramatic quantifiable absolute PF changes occurring upon cAMP excess
removal match quite well with those residues characterized by high PFs in the cAMP-bound form \((\log_{10}\text{PF}_{\text{cAMP-Bound}} > 6; \text{Fig. 2a})\). This class of highly protected core residues is confined exclusively within the CBD-A \(\beta\)-subdomain, in marked contrast with the \(\alpha\)-subdomain in which most of the residues exchange rapidly with the solvent (Fig. 2a). Within the \(\beta\)-subdomain, the residues with \(\log\text{PF}_{\text{cAMP-Bound}} > 6\) map well to the C-terminus of strands \(\beta_{1,2}\) and to most of the amides in the \(\beta\)-barrel inner strands \(\beta_{3,4}, \beta_{7,8}\) (Fig. 2a), which in turn match well with local minima of the SASA plot (Fig. 2c). Consistently with the deep burial within the protein of these core amides with \(\log\text{PF}_{\text{cAMP-Bound}} > 6\) (Fig. 2a,c), it has been previously reported that the exchange pathways for the residues with maximal protection factors often require transient global unfolding (34-36). In order to verify whether this result applies also to our system we calculated the average free energy change of the opening transition to exchange-competent states \(\Delta G_{\text{opening}}\) for the residues with \(\log\text{PF}_{\text{cAMP-Bound}} > 6\) and then we compared it to that of global unfolding \(\Delta G_{\text{unfolding}}\) measured independently by urea denaturation of the cAMP-bound form (14, 37).

For the \(\Delta G_{\text{opening}}\) computation, we considered that unfolding of the R-subunit has been previously adequately described using a two-state model (14) and that at pH 6.5 an EX2 Linderström-Lang mechanism generally applies (36, 38). As a result, the average protection factor for residues with \(\log\text{PF}_{\text{cAMP-Bound}} > 6\) (i.e. \(6.6 \pm 0.5\)) leads to
Figure 2: Protection Factors of R1α (119-244) based on H/D and H/H exchange. (a) Protection factors (PFs) derived from H/D exchange rates measured for R1α (119-244) with (black) and without (green) tenfold excess cAMP at 306 K and in 50 mM MES pH 6.5, 100 mM NaCl and 0.02% NaN₃. Filled circles refer to amide protons that exchange slowly enough to enable the measurement of quantitative H/D exchange rates; empty circles denote amide protons that were fully exchanged within the dead time of the experiment (~20 minutes); star symbols indicate amide protons that could only be detected in the first HSQC spectrum acquired after exposure to D₂O, but not in the subsequent spectra. The triangles in the lower part of panel (a) indicate the presence of hydrogen bonds donated by the backbone amide hydrogen. Triangles filled in black refer to hydrogen bonds with H---O distance < 2.40 Å and H-N...O angle < 35°, whereas open triangles indicate hydrogen bonds fulfilling less severe geometric criteria (H---O distance <
Red triangles represent inter-molecular (protein-cAMP) hydrogen bonds. The arrow at H63 signifies that for this residue no significant HSQC intensity change was observed during the course of the H/D experiment and therefore the reported PF value should just be interpreted as a PF lower limit. The arrow at Y244 means that for this residue a bi-modal behavior is observed in the presence of ten-fold excess cAMP: while most of the Y244 HSQC cross-peak intensity is lost in the first HSQC spectrum, a small but detectable residual signal remains until the completion of the H/D experiment.

Residues for which no symbol is reported are ambiguous because of overlap or are Prolines (P153 and P208).

(b) Protection factors (PFs) derived from H/H exchange rates measured under the same conditions reported for panel (a). The green/black color coding is the same as in the panel (a). Residues 132, 144 and 188 exchange fast enough to result in detectable H/H exchange CLEANEX cross-peaks only in the absence of cAMP excess (green). Residues marked with a star symbol could not be unambiguously assigned in the CLEANEX spectrum due to overlap.

(c) Residue specific solvent accessible surface areas (SASAs) for the bound form. The black solid line indicates the total (backbone and side-chain) SASAs, while the red line refers to the backbone only SASAs. The violet line refers to SASAs for backbone N atoms scaled up by a factor of 10. The reported SASA values should be considered as upper limits since the shielding effect of tightly bound water molecules as well as of cAMP is not accounted for.

(d) Differences between the protection factor logarithms measured with and without cAMP excess, shown in both panels (a) and (b) with black and green color coding, respectively.

(e) As panel (d), but showing the effect of cAMP excess removal as percentage variations in the protection factor logarithms (i.e. 100*ΔlogPF/logPF_bound). In all panels the dotted lines represent the secondary structure expected for RIA (119-244) based on the coordinates of the cAMP-bound regulatory subunit (6). Positive dots indicate α-helices or turns, whereas negative dots denote β-strands. The gray shadings indicate the residues with log_{10}(PF) > 6 in the cAMP bound form, i.e. class c residues as defined in the text. In panels (d,e) star symbols denote residues that exchange too fast in the absence of excess cAMP for a quantitative PF determination by H/D exchange or too slow in the presence of bound cAMP for the detection of H/H exchange cross-peaks.

\[ \Delta G_{\text{opening}} \approx -\text{RT} \ln(10^{6.6\pm0.5}) = 9.2 \pm 0.7 \text{ kcal/mol}. \]  

This \( \Delta G_{\text{opening}} \) estimation is in good agreement with the \( \Delta G_{\text{unfolding}} \) values measured independently by urea denaturation for cAMP-bound RIA (119-244) (i.e. 10.1 \pm 0.5 \text{ kcal/mol}) and also for other related R-subunit constructs in the presence of excess cAMP (i.e. 9.2 \pm 0.2
kcal/mol) (14, 37), further corroborating that global unfolding controls the opening transitions underlying the exchange behavior of residues with logPF\textsubscript{cAMP-Bound} > 6. This group of amino acids will be denoted here as “class c” residues, where ”c” refers to their concerted exchange behavior ensuing from transient global unfolding events. Given the collective nature of the global unfolding opening transitions that account for class c residues, we hypothesized that their PFs are similarly affected by the removal of cAMP. Indeed, this hypothesis on the concerted response of class c residues to cAMP-release is confirmed by the observation that the Δlog\textsubscript{10}PF values reported in Fig. 2d for class c amino acids are well clustered around a value of 2.0. Their standard deviation is only 0.3, which is comparable to the error already implicit in the intrinsic exchange rates employed for the PF computation (39, 40). The consistent two-order of magnitude reduction in PF values observed upon cAMP dialysis is also in agreement with the decrease in the ΔG\textsubscript{unfolding} independently revealed by urea denaturation after stripping cAMP (14), thus further corroborating that the exchange of class c residues relies on global unfolding events.

3.1.3 A “three-shell” model for the intra-molecular cAMP-dependent signalling networks.

The different results discussed above based on the cAMP-induced variations in H/H-H/D PFs and chemical shifts are accounted for here in a unified manner through a “three-shell” allostERIC model, which we propose for the intra-molecular cAMP-dependent signalling networks of CBD-A. In the context of this model, the
ensemble of contacts that radiate out from the PBC and the contiguous strands propagating the cAMP signal beyond the cAMP-binding site will be collectively referred to here as the "primary shell" of cAMP-dependent interactions (Fig. 4a). The 2D contact map shown in Fig. 3c reveals that all the sites that are most sensitive to the cAMP-dependent long-range effects as judged based on the compounded chemical shift variations (i.e. "hot spots", Fig. 3b) are accounted for by the "primary shell" of contacts radiating directly from the PBC. Two of these sites, i.e. the α-B’ helix and the C-αelix, fall within regions that are in direct contact with the C-subunit and are consistent with the N-terminal capping of the α-B’ helix by the cAMP phosphate (6) as well as with the E200/R241 electrostatic switch (9) and/or the hydrophobic hinge (11, 12) (Fig. 4a). However, some key sites that are clearly affected by cAMP based on chemical shift mapping are not involved in direct contacts with the C-subunit. Even though these cAMP-dependent interactions within the "primary shell" do not lead directly to C-binding sites, they still have the potential to contribute to the control of C-recognition by functioning as relay points that propagate the cAMP signal beyond the first ‘wave’ of contacts. These sites include the conserved D170 and I163, located at the N-terminus of the β3 strand and at the C-terminus of the β2 strand, respectively (Fig. 4a). The major effect of cAMP on the D170 region is accounted for by a salt bridge between its carboxylate and the guanidinium group of R209, which also anchors the equatorial exocyclic oxygen of the cAMP phosphate (6). In addition, the guanidinium of R209 is hydrogen-bonded to the backbone carbonyl oxygen of N171 (Fig. 4a) while the N171 amide hydrogen-
Figure 3: Effect of cAMP on Rlα (119-244) based on chemical shift changes. (a) Representative expansion of the $N_e$-exchange spectrum (orange) overlaid to the HSQC spectra of cAMP-free (blue) and cAMP-bound (green) Rlα (119-244). Dashed boxes highlight the $N_e$ exchange cross-peaks that correlate the free and bound states. The labels indicate cross-peak assignments and the lower case "f" denotes the assignments of the free form. (b) Plot of the compounded $^1H$, $^{15}N$ chemical shift changes vs. residue number. The solid and dashed horizontal lines mark the average compounded $^1H$, $^{15}N$ chemical shift ± the standard deviation, respectively. Residues for which the observed compounded $^1H$, $^{15}N$ chemical shift is larger than the average + the standard deviation (i.e. "hot spots") are highlighted with a gray background. Down/up pointing arrows indicate that the reported compounded ppm change is only an upper/lower limit.
respectively, due to either spectral overlap or the assignment of only one N\textsubscript{\text{e}} exchange cross-peak. Residues for which no data is shown are ambiguous or are Prolines (P153 and P208). The secondary structure is reported as in Fig. 2. (e) Contact map for R\textalpha (119-244) overlaid to the sites with most significant compounded chemical shift changes. A black (white) pixel is shown for a given residue pair if the minimum distance between two atoms of these two residues is < 3 Å (> 5 Å). For all other cases the color of the pixel is interpolated within a gray scale. In the top-left side of the diagonal only backbone-to-backbone distances are shown, while in the bottom-right side of the diagonal all distances are considered. The \alpha-helices are clustered along the diagonal, while the \beta-strands contacts within the \beta-barrel are represented by the two anti-diagonals. The red transparent square indicates the phosphate binding cassette (PBC), while the red dots along the diagonal correspond to sites with compounded \textsuperscript{1}H, \textsuperscript{15}N chemical shift > average + one standard deviation. The green dots indicate regions with compounded \textsuperscript{1}H, \textsuperscript{15}N chemical shift between the average + one standard deviation and the average. The size of the red and green dots takes into account that chemical shift changes report on perturbations not only in the residue for which the change is observed but also in its proximity. The red (green) solid lines connect the red (green) sites to the PBC or neighboring residues through direct inter-residue contacts. The dashed lines indicate selected contacts that are not directly involved with the PBC.

bonds the carboxylate of D170 (Fig. 4d). The backbone carbonyl oxygen of D170 is in turn hydrogen bonded with the backbone amide of R226 at the N-terminus of the B-helix, which is part of a second shell of contacts that relay the cAMP signal (Fig. 4b,d). Specifically, the enhanced solvent exposure observed for D170 in the absence of cAMP (Fig. 2b,e) is consistent with the increased flexibility required for the B-helix hinge motions necessary to bind the C-subunit (Fig. 4e).

The D170 relayed signalling pathway explains also why the D170A mutant of R is non-allosteric and is able to inhibit the C-subunit stoichiometrically even in the presence of cAMP (17). However, since it is known that C-binding causes a global re-organization of the \alpha-subdomain (7), additional intra-molecular signalling
Figure 4: Intra-molecular signalling networks that propagate the cAMP signal from the PBC to the sites of C-recognition. Panels a-c display the progression from the first to third contact shell residues, respectively, as explained in the text. Amino acids in the PBC or directly contacting it (first shell) are shown in red, those in the second shell in orange and those in the third shell in green. For first and second shell residues bonds and van der Waals surfaces are displayed, while for third shell residues detailed bonds are shown only if they are at the interface between the α- and the β-subdomains. Selected hydrogen bonds and salt bridges are indicated by thick dotted lines and regions that interface with the C-subunit are defined by dashed contours. Selected secondary structure elements and residues are labeled. Residue labels match the color of the contact shell to which they belong. Residue labels in parenthesis are only hypothetical relay sites, as discussed in the text. All figures were created using the program MOLMOL (41) and atomic coordinates from the PDB entry 1RGS (6). (d-f) The interactions of the primary shell are correlated with the conformational changes that are induced by the binding of the C-subunit. Panel (d) shows the interactions that are favoured in the cAMP bound conformation. The B/C helix is shown as a red ribbon. Highlighted
are the “electrostatic switches” (D170 and R241) that respectively anchor β3 and the C-helix to the PBC, while I163 shields the methylenes of R209. (e) Similar to panel d with the C-bound conformation (dark green) overlapped with the cAMP-bound conformation. (f) The changes in the positions of D170 and E200 in the two conformational states are highlighted.

Pathways should be considered to explain how conformational plasticity is introduced at multiple sites within the α/β-subdomain interface by the release of cAMP. For instance, another possible signalling pathway might be controlled by I163, which is the other major cAMP-dependent locus revealed through Nz-spectroscopy. I163 is in close contact with the methylenes of the R209 side chain and together with V162, is part of a previously identified conserved hydrophobic layer at the C-terminus of the β2 strand which has evolved to protect the cAMP phosphate from attack by phosphodiesterases (1) (Fig. 4b).

Both V162 and I163 belong also to an extended core of class c residues that in the presence of cAMP exchange concertedly through transient global unfolding events and that include several other amides with logPF \( _{cAMP\text{-Bound}} > 6 \) mainly clustered in the inner strands of the β-sandwich (3, 4 and 7, 8) (Fig. 2a). It is, therefore, possible that the local perturbation at the conserved V162/I163 sites initially caused by cAMP-removal has a more extensive concerted effect that contributes to collectively perturbing the whole set of class c interactions and promotes partial but global unfolding for the other class c residues as confirmed by Fig. 2d, including amino acids at the α/β-subdomain interface, such as F172, V174, I224 and W222 (Fig. 4c, tertiary shell). While other loci may also contribute to
promoting partial global unfolding in a cAMP-dependent manner, the 1163 relay site is conveniently activated by cAMP through interactions with the same highly conserved PBC residue that activates D170, i.e. R209 which, in turn, contacts the cyclic phosphate (Table S1) defining a pervasive allosteric network nucleated by cAMP binding (Fig. 4). Conversely, release of cAMP breaks this extended circuitry relayed by the sites centred at 1163 and D170 and uncouples the α- and β-subdomains from each other. In addition, the loop between 1163 and D170 includes also other key residues, i.e. the highly conserved Gl66 (1), which is hydrogen-bonded to the carbonyl of R209 (Table S1), as well as Gl69, which is critical for the cAMP-mediated activation of PKA as indicated by genetic screening (18), further reaffirming the pivotal role of this turn region for allostery.

We predict that the proposed “three-shell” model will be relevant not only for the control of the kinase activity in the PKA system but also for other homologous CBDs coupled to diverse functions. Furthermore, this work demonstrates how a small ligand such as cAMP can allosterically alter protein-protein interactions, thus opening new perspectives in drug design.
3.2 Materials and Methods

3.2.1 Sample Preparation.

The regulatory subunit of PKA (119-244) was expressed and purified based on previously described protocols (as described in chapter 2) (8). The $^{15}$N labeled protein was expressed in 2 L of $^{15}$N enriched minimal media (Spectra 9, Spectra Stable Isotopes). For all NMR samples the purified protein was dissolved in 50 mM MES pH 6.5, 100 mM NaCl and 0.02 % NaN$_3$.

3.2.2 NMR Spectroscopy

A Bruker AV 700 spectrometer equipped with a TCI cryo-probe was used for the acquisition of all NMR data. The probe temperature was set at 306 K resulting in well-dispersed HSQC cross-peaks. The $N_z$ exchange experiments (42 - 44) were run with an $N_z$ mixing period of 230 ms using a sample which contained detectable amounts of both free and cAMP-bound R1$\alpha$ (119-244). However, since the pure cAMP-free state of R1$\alpha$ (119-244) is only poorly stable in solution under the experimental conditions used, the cAMP-induced variations in hydrogen exchange rates were measured by perturbing the binding equilibrium just slightly, i.e. by dialysing out the excess free-cAMP under native conditions so that only minor populations of the cAMP-free state would co-exist in dynamic equilibrium with the bound form of the protein (45). The low concentration of the free state as well as the dynamic exchange of the cAMP ligand between the free and bound proteins ensures
the stability of these samples during the measurement of the hydrogen exchange rates (45). The H/D exchange was monitored in real-time by a series of HSQC spectra. The first 30 HSQC experiments acquired after exposure to D₂O accumulated only two scans per serial file minimizing the total acquisition time per HSQC to ~10 minutes and consequently leading to a better sampling of the fast decaying amides. For the subsequent decay the number of scans was doubled to four and 44 additional HSQC spectra were recorded. The H/H exchange rates between water and the backbone amide protons were measured using a CLEANEX-PM-FHSQC pulse sequence (46, 47) with mixing times of 5 ms, 10 ms, 30 ms, 40 ms, 60 ms and 80 ms. Further details are available as Supplementary Material.

3.3 Acknowledgments: We are grateful to CIHR, McMaster and CFI for financial support to GM and to the National Institute of Health (GM34921) to SST. GM is also grateful to the Heart and Stroke Foundation of Canada for a New Investigator award.

3.4 Foot Notes: Supplementary information includes further details for the Materials and Methods and Figures S1 and S2.

3.5 References


3.6 Supplementary Material

3.6.1 Material and Methods

3.6.1.1 Expression, Purification and NMR Sample Preparation of RIα (119 – 244)

The protein expression, purification and preparation of sample A and B are the same as described in chapter 2.

The sample of RIα (119-244) used for the N\textsubscript{2} experiment, which requires detectable amounts of free protein, was prepared through an unfolding/refolding protocol: 8 \text{ M} urea was added to sample A and it was dialyzed against 500 \text{ mL} of a 6 \text{ M} urea solution for 18 hours with three changes of the dialysis buffer at 6 hour intervals. The urea concentration was brought down to 2 \text{ M} by stepwise addition of the NMR buffer solution, while incubating on ice and stirring occasionally. The partially refolded protein was then dialyzed against 1 \text{ M} and 0.5 \text{ M} urea solutions, respectively, for 4 hours each time. The protein was finally dialyzed against 50 \text{ mM} MES pH 6.5, 100 \text{ mM} NaCl and 0.02\% NaN\textsubscript{3} to remove the urea prior to the start of the NMR experiments. This unfolding/refolding protocol resulted in a sample ("C") with detectable amounts of free RIα (119-244). After adding excess cAMP, the H\textsubscript{15}QC of this sample becomes super-imposable to that of sample B above (data not shown) indicating successful refolding of the protein.
3.6.1.2 NMR spectroscopy

The probe temperature was calibrated using a thermocouple as well as an ethylene glycol sample. For the $^{15}\text{N}$ and $^1\text{H}$ dimensions 128 and 512 complex points and spectral widths of 31.8 and 14.2 ppm were employed, respectively, unless otherwise specified. In all spectra, the $^1\text{H}$ and $^{15}\text{N}$ carrier frequencies were centered on the water resonance and in the middle of the amide $^{15}\text{N}$ region, respectively. In all experiments, $^{15}\text{N}$ decoupling during $^1\text{H}$ acquisition was obtained through a GARP pulse train with a 1.32 kHz RF pulse strength. A 100-point sine-bell shape was used for all pulse-field gradients. The Xwinmr (Bruker Inc.) or NMRPipe (2) programs were employed for spectral processing. Phase shifted squared sine bell window functions and zero filling were applied in both dimensions, unless otherwise indicated. The Gaussian line fitting protocol implemented in Sparky 3.111 (3) was used for the measurement of the cross-peak fit heights, unless otherwise specified. Estimations of the fit heights errors were calculated based on the standard deviation of the cross-peak intensity difference distribution obtained from replicate spectra, as previously explained (4). $^1\text{H}$ ppm values were calibrated using DSS, whereas the $^{15}\text{N}$ chemical shifts were indirectly referenced through the $\gamma_{N}/\gamma_{H}$ ratio (5). 2D-HSQC experiments were carried out with 128 dummy scans, 8 scans and an inter-scan delay of 1 s using sensitivity, gradient and water-flip back enhanced pulse sequences, unless otherwise specified. Standard triple-resonance experiments were used to assign the backbone resonances of cAMP-bound R1α (119-244) as previously indicated (6).
3.6.1.3 $N_z$ Exchange

When the exchange between the free and bound states occurs in the ms time-scale, the binding-induced chemical shift changes can be probed either indirectly by variations in the relaxation dispersion resulting from slight perturbations of the binding equilibrium (7) or more directly by $N_z$-exchange spectroscopy. The latter approach requires higher concentrations of the poorly soluble cAMF-free protein as compared to the relaxation dispersion method and therefore it relies on less stable samples, however when the $N_z$-spectrum can be acquired with sufficient sensitivity and resolution it provides a more direct determination of the binding induced chemical shift changes. Here, the HSQC cross-peaks for the free state of R1α (119-244) were assigned through $N_z$ exchange spectra (8, 9) based on the assignment of cAMP-bound R1α (119-244). For the acquisition of the $N_z$ exchange data sample "C" was employed. The relaxation delay between subsequent scans was 2 s and 128 scans were accumulated per $t_1$ transient after 128 dummy scans leading to a total acquisition time of $\sim$21.5 h. Some aggregation was noticed during the acquisition of the $N_z$-spectrum, however a control HSQC spectrum acquired on a 10-fold diluted sample indicates that the partial precipitation does not significantly affect the measured chemical shift changes. The resolution along the indirectly detected dimension was enhanced through semi-constant time frequency labelling (10) as well as through linear prediction. The compounded $^1H,^{15}N$ free-bound chemical shift
variation was computed as \((\Delta \delta_{1H})^2 + (\Delta \delta_{15N}/6.5)^2\)^{1/2}, as previously explained (11).

3.6.1.4 H/D Exchange

The sample for H/D exchange was prepared by passing the concentrated (1 mM) sample A through a Sephadex G10 column pre-equilibrated with 50 mM MES pH 6.5, 100 mM NaCl in 100% D_2O at 4°C. The dead time for the H/D exchange was reduced by pre-optimizing the HSQC acquisition parameters on a sample in the same buffer and with similar height as that in 100% D_2O. The measurement of the H/D exchange rates was repeated after adding 1 mM excess cAMP to both sample A and the pre-equilibration buffer. A 3 x 3 matrix centred at peak maxima was used for the quantification of the HSQC intensities with NMRPipe (2) and the spectral noise standard deviation was used to derive the uncertainties in the cross-peak intensities (4). The H/D exchange rates were obtained through the program Curvefit (12) by implementing a Levenberg-Marquardt non-linear least squares exponential fitting. Estimates of the uncertainties in the fitted decay rates were obtained both from the covariance matrix and from Monte Carlo simulations (12). Protection factors (PFs) were computed as previously described (13) assuming an EX2 Linderstrom-Lang mechanism since the pH is 6.5. The intrinsic exchange rates required for the computation of the PFs were computed using the program SPHERE implemented with a rate basis from alanine oligopeptides (14, 15). If a rate basis from poly-DL-
Ala is used, the intrinsic exchange rates decrease by about 50% resulting in a constant offset of ~0.3 in the logPF values reported in Fig. 2 (14, 15). The errors in the logPF values were determined by error propagation based on the experimental uncertainty for the measured H/D exchange rates.

3.6.1.5 H/H Exchange

A total of 32 scans were accumulated per serial file with an inter-scan delay of 2 s and a RF strength of 6.9 kHz for the CLEANEX-PM (16, 17) mixing block, which minimizes offset-effects. Replicate spectra were acquired for the short mixing times (5 ms and 10 ms). Despite the co-addition of the duplicate CLEANEX-PM-HSQC data sets at short mixing times, for selected slow exchanging residues, detectable CLEANEX cross-peaks appeared only at longer mixing periods. Build-up curves were analyzed as previously described using an FHSQC spectrum to determine reference peak heights (16, 17). Protection factors from H/H exchange rate were computed as \( \frac{k_{\text{intrinsic}}^{H/D}}{k_{\text{observed}}^{H/D}} \). The intrinsic exchange rates were computed as for the case of H/D exchange but using H2O as solvent rather than D2O in the implementation of the SPHERE computation (14, 15).

3.6.1.6 Structural and Chemical Shift Analyses

For all structure-based analyses the coordinates of the (119-244) fragment of the Rlα (91-376) crystal structure (pdb code 1RGS) were used because the 1RGS-based computed Cα and Cβ chemical shifts correlate very well with the
corresponding values measured for Rlα (119-244) in solution (Fig. S1). The Cα and Cβ chemical shifts were computed through the program ShiftX (18, 19) based on the (119-244) fragment of the 1RGS structure (20). The correlation coefficients are 0.969 and 0.994 for the Cα and Cβ chemical shift plots (Fig. S1), respectively, which are in the range expected based on the accuracy of the chemical shift prediction and on the resolution of the 1RGS structure (2.8 Å) (18, 19). Furthermore, the theoretical vs. experimental root mean square deviations are 0.773 ppm and 0.883 ppm for the Cα and Cβ chemical shifts correlations, respectively, which are well within the error of the chemical shift computation (i.e. 0.98 ppm and 1.10 ppm for Cα and Cβ atoms, respectively) (18, 19).

Hydrogen bonds and inter-residue contacts were analyzed using Molmol (21) and the 1RGS (119-244) structure (20) with computed hydrogen atoms. Solvent accessible surface areas (SASAs) were computed using the program “Getarea” (22) applied with a probe radius of 1.4 Å. The shielding effect of tightly bound water molecules and of cAMP was not considered. The secondary structure elements were identified based on the hydrogen bonding patterns according to the Kabsch/Sander algorithm (23). The local root mean square deviations (RMSDs) between the cAMP-bound (20) and the C-bound structures of Rlα (119-240) (24) were computed for each residue after superimposing the backbone heavy atoms for the amino acid triplet formed by the selected and the two adjacent residues. Residues 240-244 were excluded from this analysis due
their intrinsic flexibility which would bias the local RMSD computation. The pairwise RMSD for the backbone heavy atoms of all three residues was then calculated. Considering that the local RMSD is calculated between residue triplets, an error margin of ± one residue should be considered in the local RMSD interpretation. To eliminate local RMSD biases caused by intrinsic variability we repeated the local RMSD computation after replacing the cAMP-bound structure of the R-subunit solved at 2.8 Å resolution with the structure of the same R-construct but bound to the cAMP agonist Sp-cAMPS and solved at higher resolution (2.3 Å) (25). The Sp-cAMPS-bound analysis confirmed the pattern for the major C-induced conformational variations identified above using the cAMP-structure with the exception of the W188 site, which as suspected may be affected by intrinsic variability.

3.7 References for Supplementary Material


Figure S1: Structural assessment of R1α (119-244) based on the correlation between measured and computed Ca (a) and Cβ (b) chemical shifts.

Figure S2: Differences between the cAMP- and C-bound structures of R1α (119-244) (20, 25) in terms of local RMSDs mapped into the structure of cAMP-bound R1α (119-244) (20). The following colour codes were used: residues in dark blue are characterized by local RMSDs greater than the average + one standard deviation, while residues in light blue correspond to local RMSDs between the average and the average + one standard deviation. All remaining residues are coloured in light grey. Residues that interact
directly with the C-subunit (25) are marked by a sphere and define three major regions of C-interaction (i.e. α-B′, C-helix and the α-Xβ/A-helix loop) indicated by black dashed lines. Sites that do not interact with the C-subunit directly but are subject to significant conformational changes upon C-binding are highlighted with red lines. The W183 region is not highlighted because conformational changes at this site may, at least in part, be accounted for by C-independent intrinsic variability as explained in the Supplementary Material (See text). Selected secondary structure elements and residues are labelled. cAMP is shown as an atom-type colour coded CPK model. All figures were created using the program MOLMOL (21) and atomic coordinates from the PDB entry 1RGS (20).
Table S1: PBC Hydrogen Bonds

<table>
<thead>
<tr>
<th>Residue</th>
<th>Intra-Molecular Hydrogen Bonds</th>
<th>Inter-Molecular Contacts(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tight(^1)</td>
<td>Loose(^2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO: R209 Gua.(^3)</td>
</tr>
<tr>
<td>G199</td>
<td>--</td>
<td>CO: I204 NH</td>
</tr>
<tr>
<td></td>
<td>NH: E200 OE1</td>
<td>OE1: L201 NH</td>
</tr>
<tr>
<td></td>
<td>CO: I204 NH</td>
<td>CO: L203 NH</td>
</tr>
<tr>
<td></td>
<td>OE2: R241 Gua.(^3)</td>
<td>CO: I204 NH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NH: E200 OE1</td>
</tr>
<tr>
<td>E200</td>
<td>CO: Y205 NH</td>
<td>CO: G206 NH</td>
</tr>
<tr>
<td></td>
<td>NH: E200 CO</td>
<td>CO: T207 NH</td>
</tr>
<tr>
<td>L201</td>
<td>CO: R330 Gua.(^3)</td>
<td>NH: E200 CO</td>
</tr>
<tr>
<td>I204</td>
<td>NH: E200 CO</td>
<td>--</td>
</tr>
<tr>
<td>Y205</td>
<td>NH: L201 CO</td>
<td>--</td>
</tr>
<tr>
<td>G206</td>
<td>--</td>
<td>NH: A202 CO</td>
</tr>
<tr>
<td>T207</td>
<td>--</td>
<td>NH: A202 CO</td>
</tr>
<tr>
<td>P208</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>R209</td>
<td>CO: G166 NH</td>
<td>Gua(^3): D170 NH</td>
</tr>
<tr>
<td></td>
<td>NH: D167 CO</td>
<td>Gua(^3): G199 CO</td>
</tr>
<tr>
<td></td>
<td>Gua(^3): N171 CO</td>
<td></td>
</tr>
<tr>
<td>A210</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>NH: Water 701(^4)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) With H---O distance < 2.40 Å and H-N---O angle < 35° in the 1RGS structure (20). These criteria may not be fulfilled by all salt bridges. \(^2\) With 2.85 Å ≤ H---O distance < 2.40 Å and 35° ≤ H-N---O angle < 47° in the 1RGS structure (20). These criteria may not be fulfilled by all salt bridges. \(^3\) Guanidinium. \(^4\) From reference (25). The water molecule W701 is also hydrogen bonded to one of the cAMP Exocyclic oxygen atoms.
A MODEL FOR AGONISM AND ANTAGONISM IN AN ANCIENT AND UBIQUITOUS cAMP- BINDING DOMAIN

CHAPTER 4
Chapter Four Preference

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I conducted all of the experiments and data analysis described in this chapter.
Abstract

The cAMP-binding domain (CBD) is an ancient and conserved regulatory motif that allosterically modulates the function of a group of diverse proteins thereby translating the cAMP signal into a controlled biological response. The main receptor for cAMP in mammals is the ubiquitous regulatory (R) subunit of protein kinase A (PKA). Despite the recognized significant potential for pharmacological applications of CBDs, currently only one group of competitive inhibitor antagonists is known: the Rp-cAMPS family of phosphorothioate cAMP-analogs, in which the equatorial exocyclic oxygen of cAMP is replaced by sulfur. It is also known that the diastereoisomer Sp-cAMPS with opposite phosphorus chirality is a cAMP agonist, but the molecular mechanism of action of these phosphorothioate analogs is currently not fully understood. Previous crystallographic and unfolding investigations point to the enhanced CBD dynamics as a key determinant of antagonism. Here, we investigate the Rp- and Sp-cAMPS bound states of R(CBD-A) using a comparative NMR approach which reveals a clear chemical shift and dynamic NMR signature, differentiating the Sp-cAMPS agonist from the Rp-cAMPS antagonist. Based on these data we have proposed a model for the Rp-/Sp-cAMPS antagonism and agonism in terms of steric and electronic effects on two main allosteric relay sites.
and D170, respectively, affecting the stability of a ternary inhibitory complex formed by the effector ligand, the regulatory and the catalytic subunits of PKA. The proposed model not only rationalizes the existing data on the phosphorothioate analogs and on the CBD allostery, but it will also facilitate the design of novel cAMP antagonists and agonists.

The cAMP-binding domain (CBD)\(^1\) represents a conserved regulatory motif that modulates the function of a diverse group of proteins, including protein kinase A (PKA) in eukaryotes (1), transcription factors in bacteria (catabolite-activator protein, CAP) (2-11), guanine exchange factors (EPAC) (12-15) and ion channel proteins (both hyperpolarization-activated cyclic nucleotide-dependent channels [HCN channels] and cyclic nucleotide-gated channels [CNG channels]) (16-17). All CBDs share a conserved architecture with a contiguous \(\beta\)-subdomain nested within a non-contiguous \(\alpha\)-subdomain (18-19). The \(\beta\)-subdomain consists of an eight stranded \(\beta\)-barrel, where the cAMP phosphate binding cassette (PBC) is located. Upon cAMP binding, its signal is propagated through the \(\alpha/\beta\)-subdomain interface causing a re-arrangement of the orientation of the three \(\alpha\)-helices in the \(\alpha\)-subdomain. It is this \(\alpha\)-subdomain re-arrangement that ultimately controls the activity of the functional unit coupled to cAMP (20).

Given the significant physiological relevance of CBDs, the potential of this domain as a pharmacological target has been the subject of extensive investigations (21-23). For instance, for PKA, which is the major receptor of cAMP in vertebrates, over the past ~40 years hundreds of compounds were tested as competitive inhibitors of cAMP for
therapeutical purposes. Yet, only one group of cAMP surrogates, the phosphorothioate Rp-cAMPS analogs (Fig. 1b), was found to have an antagonist function (24-26). In Rp-cAMPS the equatorial exocyclic oxygen of cAMP is replaced by a sulfur atom, thus introducing chirality at the phosphorus position. Switching to the opposite chirality by placing the sulfur atom in the axial exocyclic position leads to the related diastereomeric cAMP-analog: Sp-cAMPS (Fig. 1a). Unlike Rp-cAMPS, Sp-cAMPS behaves as a cAMP-agonist revealing the stringent stereo-specific requirements of antagonism. However, it is currently not fully understood in molecular terms why the Rp- and Sp-cAMPS analogs function as a cAMP-antagonist and agonist, respectively.

The crystal structures of the regulatory subunit of PKA (R) bound to both phosphorothioate analogs have been solved at 2.3 Å resolution and are overall very similar to each other (i.e. Cα RMDS of 0.34 Å) and to the cAMP-bound state (i.e. Cα RMSD 0.5 Å) as well (27). Despite the overall similarity of these three structures, subtle local conformational differences were found for the conserved arginines in the PBC (i.e. R209 for CBD-A and R333 for CBD-B). For example, the inter-molecular distances indicate that the phosphothioate – R209 guanidinium interaction is significantly tighter for Rp-cAMPS as compared to Sp-cAMPS and cAMP, even though the affinity of the Rp-surrogate for this CBD is lower than that of the Sp-analog and of cAMP (27). In addition, the crystallographic investigation revealed that the Rp-cAMPS bound state is likely more dynamic than the Sp-cAMPS- and cAMP-bound forms based on its higher B-factors (27). It has also been shown that the Gibbs free energy of urea unfolding of Rp-cAMPS-bound R is similar to that of cAMP-free R, while the thermodynamics of
unfolding for Sp-cAMPS compares well with that of the more thermodynamically stable cAMP-bound R (28).

Here, we further investigate by NMR the effects of Rp- and Sp-cAMPS binding. For this purpose we focus on the 119-244 RIα fragment, which maps to CBD-A of the R-subunit of PKA and represents its minimal central controlling unit (29). Using a comparative NMR strategy based on Nz-exchange spectroscopy to assign different bound states it was possible to unveil how the internal signaling pathways of CBD-A are differentially perturbed by Rp-cAMPS as compared to Sp-cAMPS and cAMP. In addition, 15N-relaxation measurements (i.e. T₁,₂, HN-NOE and relaxation-compensated constant-time CPMG NMR dispersion) combined with hydrodynamic simulations provide further insight on the sites and time-scales of the enhanced dynamics caused by Rp-cAMPS. The interpretation of these results in the context of the allosteric networks of CBD-A and of the stereo-electronic effects caused by the oxygen-to-sulfur isolobal substitution has led to the proposition of a molecular model for the antagonism and agonism of the Rp- and Sp-cAMPS analogs, respectively.

4.1 Experimental procedures

4.1.1 NMR Sample Preparation of RIα (119 – 244) Bound to Different Ligands.

Samples of uniformly 15N-labeled AMP-bound RIα (119 – 244) in MES buffer (50 mM MES pH 6.5, 100 mM NaCl and 0.02 % NaN₃) were prepared as
previously discussed in chapter 1 (29, 30). The Rα (119 – 244) samples with sub-stoichiometric amounts of cAMP used for the Nz-exchange experiments were obtained through a protocol of unfolding, partial dialyzing out of cAMP and refolding. Specifically, after adding 8 M urea to cAMP-bound Rα (119 – 244) the sample was dialyzed for 18 hours in the presence of 0.5 L MES buffer with 6 M urea and 1 mM DTT. After changing the dialysis buffer three times at regular intervals, the protein was then partially refolded by step-wise dilution of the dialysis solution to 0.5 M urea through addition of MES buffer with 1 mM DTT. Complete removal of urea was obtained by a final dialysis against the MES buffer, which resulted in a sample with NMR detectable amounts of both cAMP-bound and free states of Rα (119 – 244) at a total concentration of ~0.1 mM. Addition of 50 μM Sp-cAMPS phosphorothioate cAMP-analog (Sigma) to this sample resulted in another NMR sample with NMR observable amounts of both cAMP-bound and Sp-cAMPS-bound Rα (119 – 244). This sample was used to assign the Sp-cAMPS-bound Rα (119 – 244) through Nz-exchange spectroscopy. The sample with both cAMP-bound and Rp-cAMPS- (Sigma) bound Rα (119 – 244) was prepared similarly. Samples containing only Rα (119 – 244) bound to the Sp-cAMPS or Rp-cAMPS analogs were prepared by extensively rather than partially dialyzing the protein under denaturing condition and then refolding. Specifically, we added urea to 6 mL of 0.15 mM Rα (119-244) to a final urea concentration of 8 M. After keeping the resulting solution at room temperature for 30 min, it was dialyzed against 8 M urea buffer (50 mM MES pH 6.5, 100 mM NaCl and 1 mM DTT) for 16 hours with three buffer
changes. The protein solution was then dialyzed extensively with 6 M urea for 24 hours to completely remove cAMP. After refolding the protein as indicated above, 1 mM excess Sp-cAMPS analog was added. The Rp-cAMPS bound Rlα (119 – 244) sample was prepared likewise.

4.1.2 NMR Spectroscopy.

All NMR data were acquired using a Bruker AV 700 MHz spectrometer equipped with a TCI cryo-probe set at temperature of 306 K. The calibration of the probe temperature was obtained using a thermocouple as well as an ethylene glycol sample. Unless otherwise specified all spectral widths, number of digitization points, carrier frequencies, $^{15}$N GARP decoupling strength and gradient shapes were set as previously discussed (29). Spectra were processed using the Xwinnmr (Bruker Inc.) or NMRPipe (31) programs based on previously published standard protocols (29-32). Cross-peak fit heights were measured using the Gaussian line fitting routine implemented in Sparky 3.111 (33) and the respective error were estimated from replicate spectra as described (34). The backbone resonances of cAMP-bound Rlα (119-244) were assigned through standard triple-resonance experiments as previously indicated (35).

4.1.3 Nz Exchange.

The assignment of the backbone amides of Rlα (119 – 244) bound to Sp-cAMPS was obtained from that of cAMP-bound Rlα (119 – 244) through Nz-exchange spectra (36-38) acquired for a sample with NMR observable amounts of
both cAMP-bound and Sp-cAMPS-bound RIA (119–244). Similarly, the backbone amides of RIA (119–244) bound to Rp-cAMPS were assigned through $N_2$-exchange spectra of a sample with NMR observable amounts of both cAMP-bound and Rp-cAMPS-bound RIA (119–244). In both cases the $N_2$ mixing period was 230 ms and the relaxation delay between subsequent scans was 2 s. After acquisition of 128 scans per $t_1$ transient the $N_2$-exchange spectra were processed using linear prediction. The $((\Delta \delta_{1H_N})^2 + (\Delta \delta_{15N_H}/6.5)^2)^{1/2}$ equation (39) was used to compute the compounded $^1H,^{15}N$ chemical shift variations between the different states of RIA (119–244) (i.e. X-bound vs. free and X-bound vs. cAMP-bound, where X = Sp- or Rp-cAMPS).

4.1.4 Relaxation Dispersion NMR.

A relaxation-compensated constant-time (RC-CT) CPMG pulse sequence (40-42) was used to measure the $^{15}N$ relaxation dispersions for backbone amides in the cAMP-, the Rp-cAMPS- and the Sp-cAMPS-bound states of RIA (119-244). The total CPMG length ($T_{CP}$) was 93.3 ms with either eight or eighty-eight $180^\circ$ $^{15}N$ pulses corresponding to CPMG field strengths ($v_{CPMG}$) of 43 Hz and 472 Hz, respectively (40-41). Fifteen interleaved replicate data sets were acquired and co-added for each CPMG field strengths with 128 dummy scans and 16 scans per serial file separated by an inter-scan delay of 2.2 s. In addition, considering that at 700 MHz and at the CPMG RF strength employed (3.1 kHz) $^{15}N$ pulses are affected by significant offset-effects, all CT-RC-CPMG experiments were acquired with three different $^{15}N$ carrier frequencies (110 ppm, 119 ppm and 127 ppm) to span the $^{15}N$
spectral width using three narrow bands. The NMR relaxation dispersion ($\Delta R_2^{\text{eff}}$) was calculated as: $\Delta R_2^{\text{eff}} = R_2^{\text{eff}}(43 \text{ Hz}) - R_2^{\text{eff}}(472 \text{ Hz})$. Since: $R_2^{\text{eff}}(\nu_{\text{CPMG}}) = (-1/T_{\text{CP}}) \ln(I_{\nu_{\text{CPMG}}}/I_o)$ (40-41), where $I_{\nu_{\text{CPMG}}}$ and $I_o$ are the cross-peak intensities with and without the CPMG periods, respectively, the equation for $\Delta R_2^{\text{eff}}$ can be recast as: $\Delta R_2^{\text{eff}} = (1/T_{\text{CP}}) \ln(I_{472\text{Hz}}/I_{43\text{Hz}})$. For cross-peaks affected by overlap and/or relaxing too quickly to be detected in the RC-CT-CPMG spectra (i.e. V134, H138 and L139 in all three bound states) the $\Delta R_2^{\text{eff}}$ computation was not possible.

4.1.5 Other $^{15}$N NMR Relaxation Measurements.

The $^{15}$N spin-lattice and spin-spin relaxation rates $R_1$ and $R_2$ as well as the $\{^1\text{H}\}^{15}$N steady state NOEs were measured for the cAMP-, the Rp-cAMPS- and the Sp-cAMPS-bound states of R1α (119-244) using pulse sequences with water flip back pulses and sensitivity enhancement (34, 43-48). The $R_1$ relaxation rates were measured using relaxation delays of 100 ms (x2), 200 ms, 300 ms, 400 ms (x2), 500 ms, 800 ms, 900 ms and 1000 ms (where x2 indicates duplicate spectra). The offset- and duty-cycle compensated $^{15}$N $R_2$ CPMG experiments (49) were measured with a $\nu_{\text{CPMG}}$ of 472 Hz (40-41) and CPMG relaxation delays of 8.48 ms, 16.96 ms, 25.4 ms, 33.92 ms, 42.4 ms, 50.88 ms, 59.36 ms, 76.32 ms and 93.28 ms. The steady state $\{^1\text{H}\}^{15}$N NOE values were measured from the ratio of peak intensities determined with and without proton saturation. For the NOE experiment a 12 s recycle delay was used including a 5 s proton saturation period (43, 45), while for the $R_1$ and $R_2$ pulse sequences the recycle delay was 1.5 s. $^1\text{H}^{15}$N HSQCs spectra were collected before
and after each relaxation experiments to check the stability of the protein sample. The whole series of \( R_1 \) and \( R_2 \) relaxation rate measurements with different relaxation periods was collected in triplicate to average potential long-term instabilities (39) and for the NOE experiments ten sets of data were collected both with and without proton saturation. All replicate spectra were co-added before processing with NMRPipe (31). The relaxation rates and their errors were computed through relaxation fitting simulations with Sparky 3.111 (50) using 1000 iterations with a Gaussian-distributed random noise added. The uncertainty in the steady state NOE was measured based on the standard deviation of the distribution of the differences in fit heights between duplicate spectra (34). All errors were treated as previously explained (51). Selected cross-peaks could not be used in the relaxation analysis due to line-broadening and/or overlap. For the Rp-cAMPS-bound form some of these cross-peaks correspond to residues in the oX_{N-/}A-helix loop.

4.1.6 Reduced Spectral Density Mapping.

The \(^{15}\)N relaxation data were mapped into reduced spectral densities assuming that their high frequency values are constant: \( J(\omega_N + \omega_H) = J(\omega_H) = J(\omega_N - \omega_H) \), as previously described (52-55). Under this assumption, the \( J(\omega_N + \omega_H) \) and \( J(\omega_N) \) values are derived only from the measured \(^{15}\)N \( R_1 \) and \( \{^1\text{H}\}^{15}\)N NOEs, whereas the \( J(0) \) values are computed using the measured \(^{15}\)N \( R_2 \) rates as well (55). The calculated value of \( J(0) \) includes also chemical exchange contributions. Errors in the reduced spectra densities were assessed through propagation of the uncertainties in the experimentally determined \(^{15}\)N relaxation rates and NOEs. For reference
purposes the Lorentzian spectral density of an isotropically diffusing rigid molecule was computed as: \( J(\omega) = \frac{(2/5) \tau_c}{(1 + (\omega \tau_c)^2)}. \)

4.1.7 **Hydrodynamic Simulations.**

The contributions of the overall tumbling and the effect of diffusion anisotropy on the relaxation rates and the reduced spectral densities were modeled through bead method based hydrodynamic simulations using the HYDRONMR program (56,57). For this purpose the coordinates for the 119-244 fragment of the 1RGS PDB structure of RIo were employed with hydrogen atoms added through the program Molmol (58) and with an atomic element radius of 3.3 Å, which represents the optimal average value that has been previously found to best fit several hydrodynamic properties (*i.e.* translational diffusion, sedimentation coefficients, rotational diffusion and intrinsic viscosity) for a set of model proteins (57). An error of ± 0.2 Å was considered for the atomic element radius to account for hydration layer variability. A temperature of 306 K was used for the HYDRONMR simulation and the viscosity of water in centipoises (cP) at this temperature was computed as (57): \( \eta = 1.7753 - 0.0565t + 1.0751 \times 10^{-3} t^2 - 9.2222 \times 10^{-6} t^3, \) where \( t \) is the temperature in Celsius. The \(^{15}\)N relaxation rates at a static field of 16.44 T computed by HYDRONMR for the rigid RIo (119-244) assume an N-H distance of 1.02 Å and a chemical shift anisotropy of -160 ppm (56,57). The \( D_{par}/D_{per} \) ratio was computed as \( 2D_x/(D_x + D_y), \) where \( D_x \) and \( D_y \) are the pair of eigenvalues of the rotational diffusion matrix that are closest to each other with \( D_x > D_y \) (56,57). Similar computations were repeated for the cAMP-binding domain A of the R-subunit in
different bound forms (i.e. Sp- and Rp-cAMPS bound as well as C-subunit bound with PDB codes 1NE6, 1NE4 and 1U7E, respectively). Additional considerations for the analysis of the relaxation rates taking into account the conformational plasticity of the α-subdomain (30, 59-62) can be found in the Supplementary Material.

4.1.8 **Secondary Structure Analyses.**

The secondary structure elements of 1RGS were identified based on the hydrogen bonding patterns according to the Kabsch/Sander algorithm (63), while the solution secondary structure probabilities were predicted based on the sequence of R1α (119-244) and on the measured chemical shifts using the program PECAN (64).

4.2 Results

4.2.1 **Interaction Mapping of the Phosphorothioate Sp-cAMPS Agonist with CBD-A Using N2-Spectroscopy.**

The binding of the cAMP-agonist Sp-cAMPS (Fig. 1a) to R1α (119-244) was mapped by \(^1\)H and \(^{15}\)N chemical shift changes measured through N\(_2\)-spectroscopy (Fig. 1c) (36-38). The resulting compounded chemical shift variations are reported in Fig. 2a, c. Panel 2a shows the Sp-cAMPS binding induced compounded \(^1\)H and \(^{15}\)N chemical shift changes of R1α (119-244), while Fig. 2c reports the compounded \(^1\)H and \(^{15}\)N ppm differences between the cAMP-bound and the Sp-cAMPS-bound forms of the same R1α construct. The pattern revealed by panel 2a is similar to what has
been previously observed for cAMP-binding to RIα (119-244) (29). Statistically significant chemical shift changes are observed not only for the edges of the

Figure 1. Chemical structures of Sp-cAMPS (a) and Rp-cAMPS (b), respectively, and representative expansions from the longitudinal $^{15}$N$_{H}$-exchange spectra of RIα (119 – 244) in the presence of cAMP and Sp-cAMPS (c) and of cAMP and Rp-cAMPS (d). The auto-peaks of the cAMP-bound state are indicated by the residue number and those of the analog-bound state by Sp- or Rp- appended to the residue number. The exchange cross-peaks arising from the transfer of magnetization between the cAMP- and the analog-bound states are connected by dashed lines. (e, f) Outline of the interaction networks radiating from the sulfur atoms of the phosphorothioate analogs of cAMP. (e) Sp-cAMPS bound to site A of the regulatory subunit
of PKA (PDB code 1NE5) (27). (f) Rp-cAMPS bound to site A of the regulatory subunit of PKA (PDB code 1NE4) (27). In both panels the exocyclic sulfur and oxygen atoms of the phosphate are in yellow and red, respectively. The bound water molecule in the phosphate binding cassette is highlighted in blue in panel (e). Hydrogen bonds are depicted by black dashed lines. Selected residues are shown with red lines surrounded by their dotted van der Waals surfaces. The cAMP analogs are represented as CPK models. All images in panels (e, f) were created using the Molmol program (58).

The similarity between cAMP and the Sp-cAMPS analog in terms of binding and allosteric effects is also confirmed by the comparison between the chemical shifts of cAMP-bound and of Sp-cAMPS-bound RIα (119-244) (Fig. 2c). No significant chemical shift change is observed for the majority of the residues in RIα (119-244) and the only significant variations are localized within the PBC and at residue N185 (Fig. 2c). Most of the PBC ppm differences reported in Fig. 2c are likely the result of the slight repositioning of Sp-cAMPS relative to cAMP within the RIα (119-244) binding pocket (27) caused by the steric effects of the Sp-cAMPS sulfur atom (van der Waals radius of 1.70 Å and P-S bond length of 1.95 Å) as compared to the cAMP oxygen atom (van der Waals radius of 1.35 Å and P-O bond
length of 1.50 Å) (65). Furthermore, not only steric but also electronic effects should be taken into account for the amide of A210, which is hydrogen bonded to the sulfur atom of Sp-cAMPS both directly and indirectly through a bridging water molecule.

**Figure 2.** Compounded chemical shift map of the effects of the cAMP-agonist (Sp-cAMPS) and the cAMP-antagonist (Rp-cAMPS) on R1α (119-244). The compounded chemical shifts were calculated between (a) Sp-cAMPS bound- and free R1α (119 – 244) and (b) Rp-cAMPS bound- and free R1α (119 – 244), respectively. Panels (c, d) are similar to panels (a,b) with the free state replaced by the cAMP-bound form of R1α (119-244). For residue 210 in panel (c) the chemical shift value has been down-scaled by a 0.5.
factor to accommodate all the chemical shift changes within the same scale. In all panels the solid and dashed horizontal lines indicate the average chemical shift ± standard deviation, respectively. The residues highlighted with grey background are characterized by chemical shift variations larger than the average + standard deviation in p.nel (d). The residues for which no data is shown are either ambiguous or are prolines (P153 and P208). The down/up pointers indicate that the ppm changes represent only an upper/lower limit due to spectral overlap or the assignment of only one N\textsubscript{z} cross-peak. The secondary structure for R\textalpha\textsubscript{c} (119 - 244) is represented by the black dotted line in panel b. The rectangles defined by the red dotted lines identify the chemical shift signature that differentiates agonists from antagonists.

(Fig. 1e) (27). The dual nature of the interaction between the A210 NH and the axial exocyclic heterocatom of the ligand explains the very dramatic chemical shift change detected for A210 upon replacement of oxygen with the bulkier and less electronegative sulfur in Sp-cAMPS (Fig. 1c and Fig. 2c). In addition, inter-residue distances (Table S1) indicate that upon replacing cAMP with Sp-cAMPS, A210 is pushed away from the phosphorus atom and towards Q165, which is in van der Waals contact with A210 and hydrogen bonds the amide of N185 (Fig. 2e). The backbone NH of N185 is therefore in an ideal position for sensing this relayed long-range steric effect caused by Sp-cAMPS, explaining the significant chemical shift change observed for N185 in Fig. 2c.

4.2.2 Interaction Mapping of the Phosphorothioate Rp-cAMPS Antagonist with CBD-A Using N\textsubscript{z}-Spectroscopy.

Similarly to Sp-cAMPS, the binding of the cAMP-antagonist Rp-cAMPS (Fig. 1b) to R\textalpha\textsubscript{c} (119-244) was investigated using the \textsuperscript{1}H and \textsuperscript{15}N chemical shift changes obtained by N\textsubscript{z}-spectroscopy (Fig. 1d). The resulting compounded chemical
shift variations are reported in Fig. 2b, d. The Rp-cAMPS binding induced $^1$H and $^{15}$N chemical shift changes of RIα (119-244) are shown in Fig. 2b, while panel 2d shows the ppm differences between the cAMP-bound and the Rp-cAMPS-bound states of this cAMP-binding domain. Fig. 2b reveals a very distinct difference between the allosteric behavior of the Sp-cAMPS and Rp-cAMPS. While the binding of both phosphorothioate analogs causes perturbations at the PBC and at the more remote I163 and α-B/α-C interface regions (Fig. 2a, b), only the Sp-cAMPS agonist significantly perturbs the locus centered around D170 (Fig. 2a, b). When the Rp-cAMPS antagonist binds RIα (119-244) the D170 region remains largely unaffected as compared to the free-state.

Another clear difference between the Sp- and Rp-cAMPS analogs is revealed by the inspection of Fig. 2d comparing the cAMP- and Rp-cAMPS bound states of RIα (119-244). This figure shows that the replacement of the equatorial oxygen of bound cAMP with a sulfur atom causes significant perturbations not only at the PBC and at D170 but also in the allosteric sites centered at I163 and at the α-B/α-C boundary (29). These extensive long-range perturbations caused by the Rp-cAMPS/cAMP substitution (Fig. 2d) are in marked contrast with the trend observed for Sp-cAMPS (Fig. 2c) and are accounted for by the more extensive RIα interaction network that radiates from the equatorial sulfur of Rp-cAMPS (Fig. 1f) as compared to the axial sulfur of Sp-cAMPS (Fig. 1e). The equatorial exocyclic heteroatom (i.e. oxygen or sulfur) in cAMP and its phosphorothioate analogs is anchored by multiple
hydrogen bonds involving the carbonyl oxygen of G199, the amide of A202 and the guanidinium of R209. Perturbations at the equatorial position of cAMP-like ligands are therefore expected to cause extensive effects that propagate beyond the immediate PBC. For instance, R209 is ion paired to D170 located at the N-terminus of β3 and its methylenes are also in van der Waals contact with the side chain of I163, which is positioned at the C-terminus of β2 and is in turn adjacent to V213 found within β7 (Fig. 1f). This interaction network explains the peak variations observed in Fig. 2d not only for the PBC but also for the I163 and D170 regions. Besides, the significant ppm changes reported in the same panel (Fig. 2d) for the region at the α-B/α-C interface are consistent with the coupling between this site and the PBC occurring through the E200/R241 salt bridge (66) and/or a hydrophobic hinge involving I204, Y229 and M234 (12, 59).

Even though the analysis of the compounded chemical shift variations (Fig. 2) clearly unveils significant differences between the Sp-cAMPS agonist and the Rp-cAMPS antagonist, a full understanding of their functional diversity warrants the investigation of their dynamic behavior as well. For reference purposes, we will first characterize the flexibility of the cAMP-bound form of R1α (119-244). We will then analyze the changes in dynamics occurring when the axial or the equatorial exocyclic oxygens of cAMP are each replaced by a sulfur atom, i.e. when Sp-cAMPS or Rp-cAMPS, respectively, take the place of cAMP.
4.2.3 General Approach to the Characterization of the Dynamics of cAMP-Bound Rlα (119-244).

The ps-ns and ms-μs backbone dynamics of cAMP-bound Rlα (119-244) were characterized through the measurement of the $^{15}$N R$_1$ and R$_2$ relaxation rates and of the $\{^1H\}$-$^{15}$N-NOE data shown in Fig. 3a-d (black circles) (34, 43-49). These relaxation data were also translated into reduced spectral densities (52-55), which are reported in Fig. 4. In addition, slow (ms) motions that do not contribute significantly to the R$_2$ rates measured through traditional CPMG pulse sequences (34, 67) were probed by relaxation-compensated constant-time (RC-CT) CPMG experiments (40-41). The residue-specific relaxation dispersions ($\Delta R_{2,\text{eff}}$) quantified through the RC-CT-CPMG measurements are reported in Fig. 3e. The ps-ns and ms-μs dynamic features of R(CBD-A) are mapped into its 3D-structure in Fig. 5. The dynamics profiles defined by the $^{15}$N relaxation measurements were also related to the secondary structure probability map computed based on the observed chemical shifts (Fig. 6).

4.2.4 Dynamic Features of cAMP-Bound Rlα (119-244): The α-Subdomain.

The data of Figs. 3 and 4 clearly point to the C-helix as the most dynamic secondary structure element of Rlα (119-244) (Fig. 5ab). The $\{^1H\}$-$^{15}$N-NOE values (Fig. 3c) observed for the C-helix are significantly lower than those measured for the core residues of the α-X$_n$ and A-helices indicating that only the C-helix is dynamic in the ps-ns time scale. This helical stability trend is confirmed by the reduced spectral densities (Fig. 4). The ps-ns motions of the C-helix are consistently indicated by the
elevated values observed for the high-frequency spectral densities \( J(\omega_H + \omega_N) \) (Fig. 4c, 5ab) and by the low values measured for the spectral densities at lower-frequencies \( J(\omega_N) \) and \( J(0) \) (Fig. 4a, b), which take into account not only the \({^1H}\)-\(^{15}N\)-NOEs but the \(^{15}N\) \( R_1 \) and \( R_2 \) rates as well. In addition, a similar helix stability pattern is derived based on the secondary chemical shifts (Fig. 6). The measured secondary chemical shifts are fully compatible with a prevailing helical conformation for the residues in the \( \alpha-X_n \) and A-helices as found in the 1RGS structure (Fig. 6). In contrast, the measured secondary chemical shifts of most residues in the C-helix point to a conformational equilibrium that consists of significant populations of ‘random-coil’ conformations (Fig. 6).

Our relaxation data also indicate that, despite the relative rigidity of the \( \alpha-X_n \) and A-helices in the ps-ns time-scale, the turn connecting them is affected by slow (ms-\( \mu \)s) motions (Figs. 3de, 4a and 5cd). For instance, several residues between the \( \alpha-X_n \) and the A-helices are characterized by higher than average values observed for the \( R_1R_2 \) products (Fig. 3d) and for the reduced spectral densities at zero frequency (Fig. 4a). These \( R_1R_2 \) and \( J(0) \) variations are significantly larger than the fluctuations expected based on the anisotropy of the overall tumbling as indicated by hydrodynamic modeling (Fig. 3d, 4a, S1 and S2) and are therefore an indication of ms-\( \mu \)s motions. The ms-\( \mu \)s dynamics for the residues between the \( \alpha-X_n \) and the A-helices is also consistent with the secondary chemical shift results (Fig. 6) and with the line-broadening beyond detection of the V134, H138 and L139 cross-peaks.
Figure 3. Backbone $^{13}$N relaxation rates for RIA (119-244) bound to cAMP (black), Sp-cAMPS (green) and Rp-cAMPS (orange) are plotted against residue number. All data were measured at a field of 700 MHz in the presence of ten-fold excess of ligand at 306 K and in 50 mM MES pH 6.5, 100 mM NaCl and 0.02% NaN$_3$. The red lines in panels (a-d) denote rates computed based on hydrodynamic bead models of RIA (119-244) with an optimal atomic element radius of 3.3 Å (thick line) ± 0.2 Å (thin lines) to account for the variability in the hydration layer. (a) Spin-lattice relaxation rates ($R_1$); (b) Spin-spin relaxation rates ($R_2$); (c) $^{15}$N{$^1$H} NOE computed as $I_{\text{sat}}/I_{\text{non-sat}}$. This panel also reports schematically the secondary structure elements similarly to Fig. 2. (d) Product of $R_1$ and $R_2$. This product is only marginally sensitive to diffusional anisotropy; (e) Dispersion of effective $R_2$ relaxation rates measured from multi-offset relaxation-
compensated constant-time CPMG measurements and computed as: \( \Delta R_2^{\text{eff}} = R_2^{\text{eff}}(43 \text{ Hz}) - R_2^{\text{eff}}(472 \text{ Hz}) \), where the numbers in parentheses denote the CPMG field strengths. The red horizontal line in this panel corresponds to the absence of dispersion (\( \Delta R_2^{\text{eff}} = 0 \text{ s}^{-1} \)). Residues for which no relaxation data is reported correspond to cross-peaks which are prolines (P153 and P208) or are overlapped and/or broadened beyond detection.

The flexibility in the region of the \( \alpha-X_n / A\)-helix loop is further supported by the loose packing of the \( \alpha-X_n \) helix suggested by the rapid HD exchange observed for the indole NH of W222 (data not shown), which in the X-ray structure of R1\( \alpha \) (91-376) (PDB code 1RG9) is buried at the interface between the \( \beta \)-subdomain and the \( \alpha-X_n \) helix (18).

4.2.5 Dynamic Features of cAMP-Bound R1\( \alpha \) (119-244): The \( \beta \)-Subdomain

Our \( ^{15}\text{N} \) relaxation data (Figs. 3 and 4) point to a \( \beta \)-subdomain significantly less flexible than the \( \alpha \)-subdomain, in full agreement with previous hydrogen exchange results (30, 59). The relaxation data (Figs. 3 and 4) for most of the amides in strands \( \beta_{3,4}, \beta_{7,8} \) and at the C-terminus of strands \( \beta_{1,2} \) (Fig. 5) are consistent with the absence of significant internal ms-\( \mu \)s and ps-ns motions as confirmed by the corresponding reduced spectral density maps, which do not display major deviations from the values predicted based uniquely on the overall tumbling (Fig. 4 and S2). In this regard, it should also be noted that the discrepancy observed between the solution and the crystal secondary structure profiles in Fig. 6 for the \( \beta_2 \) strand (residues 161-163) is not likely to reflect enhanced flexibility at this locus. The difference between the NMR and X-ray based secondary structure profiles for the \( \beta_2 \)
strand (Fig. 6) is indeed consistent with the significant dihedral angle deviation from the optimal $\beta$-strand value observed for V162 \textit{(i.e. $\phi_{162} = -36^\circ$)}, explaining why it is not possible to identify the $\beta_2$ strand based on the dihedral-angle sensitive chemical shifts. Despite this phi angle distortion the hydrogen bonds defining the $\beta_2$ strand are preserved and therefore the hydrogen-bond based Kabsch/Sander algorithm \textit{(63)} clearly identifies the $\beta_2$ strand in the crystal structure coordinates.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{Reduced spectral densities for RI$\alpha$ (119-244) bound to cAMP (black), Sp-cAMP (green) and Rp-cAMP (orange) are plotted against residue number. Filled circles indicate spectral density values obtained from the experimental relaxation rates reported in Fig. 3. Red lines denote spectral density values derived from the relaxation rates computed based on hydrodynamic bead models. As in Fig. 3 thick red lines}
\end{figure}
correspond to an atomic element radius of 3.3 Å, whereas thin red lines are derived using atomic element radii of 3.1 Å and 3.5 Å. (a) Plot of J(0) values including contributions from chemical exchange effects. Secondary structure elements are shown similarly to Fig. 3(c); (b, c) Plot of J(\(\omega_N\)) and J(\(\omega_N + \omega_H\)) values, respectively, where \(\omega_N = -\gamma_N B_o\) and \(\omega_H = -\gamma_H B_o\) and B_o denotes the static magnetic field (16.44 T).

Despite the overall rigidity of the \(\beta\)-barrel, at least two main dynamic sites can be identified within the \(\beta\)-subdomain. The first \(\beta\)-subdomain dynamic site is D170 which is located at N-terminus of \(\beta_3\) and is affected by fast (ps-ns) local motions (Figs. 3c, 4c and 5ab). The second \(\beta\)-subdomain dynamic site includes the \(\beta_{5,6}\) strands and the \(\alpha\)-B’ helix within the adjacent PBC, defining a region characterized by slower (ms) and less local fluctuations (Figs. 3e, 4a and 5cd). The ps-ns motions of D170 are identified by its decreased NOE (Fig. 3c) and its elevated J(\(\omega_H + \omega_N\)) value (Fig. 4c). The ms motions in the \(\beta\)-strands 5 and 6 and \(\alpha\)-B’ helix regions are supported by the significant relaxation dispersion observed for residues T190, F198, G199 and Y205-T207 (Figs. 3e and 5cd). In addition, higher than average values are measured for both the R_1R_2 product (Fig. 3d) and the J(0) (Fig. 4a) of L203 in the \(\alpha\)-B’ helix. Since these R_1R_2 and J(0) variations cannot be accounted for by diffusional anisotropy as shown by hydrodynamic simulations (Figs. 3d, 4a, S1 and S2) they provide further indications of dynamics in the ms-\(\mu\)s time-scale. Other loci affected by ms dynamics as revealed by the NMR dispersion measurements (Fig. 3e) are located in the \(\beta\)-barrel loops such as the hyper-variable region between \(\beta_4\) and \(\beta_5\) and the \(\beta_{1,2}\) turn (Fig. 5cd).
4.2.6 **Effects on the CBD-A Dynamics of the Oxygen to Sulfur Replacements at the Exocyclic Phosphate Positions of cAMP.**

The $^{15}$N $R_1$, $R_2$ rates, the NOEs and the corresponding reduced spectral densities as well as the NMR dispersion $\Delta R_{2,\text{eff.}}$ values were measured also for RI$\alpha$ (119-244) bound to Sp-cAMPS (green circles in Figs. 3 and 4) and to Rp-cAMPS (orange circles in Figs. 3 and 4). In the case of the Sp-cAMPS-bound form (Fig. 1a, e) no dramatic changes in the dynamic features are observed relative to the cAMP-bound state discussed above (Figs. 3 and 4). However, for the Rp-cAMPS-bound form (Fig. 1b, f), variations in the slow (ms-$\mu$s) dynamic behavior for two main functional regions, *i.e.* the $\beta_{2,3}$ loop and the PBC, are identifiable relative to the cAMP- and Sp-cAMPS-bound states (Figs. 3 and 4). These differences are clearly appreciated through the 2D correlation plots of $\omega_N$ vs. $J(0)$ for the $\beta_{2,3}$ loop and the PBC segments (Fig. S3) as well as through the comparative statistical analysis of the reduced spectral densities and the NMR dispersion $\Delta R_{2,\text{eff.}}$ values summarized in Table 1.

Table 1 clearly shows that in both the $\beta_{2,3}$ loop and the PBC regions the average $J(0)$ values for Rp-cAMPS are significantly higher than those for cAMP and Sp-cAMPS. Considering that the overall tumbling correlation times and diffusional anisotropies of RI$\alpha$ (119-244) do not differ significantly among the cAMP and the phosphorothioate analog ligands (Table S2), the $J(0)$ trend revealed by Table 1 suggests the presence of enhanced ms-$\mu$s dynamics at the $\beta_{2,3}$ turn and the PBC sites.
Figure 5. Summary of multi-time scale dynamics for cAMP-bound RIα (119-244). (a, b) Map of ps-ns dynamics obtained by encoding the $J(\omega_H+\omega_N)$ values in both the width and the color of the backbone worm. Blue and yellow code for the minimum and maximum $J(\omega_H+\omega_N)$ values, respectively, while intermediate $J(\omega_H+\omega_N)$ values are linearly interpolated. Similarly the worm width is linearly proportional to the $J(\omega_H+\omega_N)$ values. (c, d) Map of ms-μs dynamics based on J(0) and NMR dispersion data. All non-ambiguous residues are colored blue unless they are considered affected by chemical exchange broadening based on the condition: $J(0) > (J(0)_{\text{average}} + \sigma_{J(0)} + \varepsilon_{J(0)})$ and/or $\Delta R_{\text{2eff}} > (\Delta R_{\text{2eff,average}} + \sigma_{\Delta R_{\text{2eff}}} + \varepsilon_{\Delta R_{\text{2eff}}})$, where $\sigma$ and $\varepsilon$ indicate the standard deviation and the experimental error, respectively. The average on J(0) was computed after excluding residues with HN NOE < 0.65. Residues subject to chemical exchange broadening based on the above criteria are painted yellow and are depicted with a doubled worm width. In all panels residues that are ambiguous due to overlap and/or line-broadening beyond detection are colored.
grey and their worm width is interpolated based on the adjacent residues. cAMP is represented as a CPK model colored according to atom type. All images were created using the program MOLMOL (58) and atomic coordinates from the PDB entry 1RGS (18). Selected residues and secondary structure elements are labeled. The regions marked by dashed lines in each panel are the main sites of interaction with the C-subunit (black dashed lines) as well as the main loci of C-induced conformational change (red dashed lines) (20).

in the Rp-cAMPS-bound state relative to the Sp-cAMPS- and cAMP-bound forms. These results are confirmed by the trend observed in Table 1 for the independently measured \( \Delta R_{2,eff} \), i.e. the average \( \Delta R_{2,eff} \) values for Rp-cAMPS are significantly higher than those computed for cAMP and Sp-cAMPS both in the \( \beta_2,3 \) loop and in the PBC regions. The enhancement of the slow (ms-\( \mu s \)) dynamics specific of the Rp-cAMPS analog is highlighted in Fig. 7 by the orange ribbon shown for the \( \beta_2,3 \) turn and the PBC in Rp-cAMPS bound R1\( \alpha \) (119-244).

No significant changes were identified in the fast (ps-ns) dynamic time scale as revealed by the absence of statistically meaningful variations in the average \( J(\omega_{H}+\omega_{N}) \) values among the three bound states for both the \( \beta_2,3 \) loop and in the PBC sites (Table 1). Similar considerations apply to the analysis of the average \( J(\omega_{N}) \) variations in the PBC (Table 1). However, a significant decrease in the mean \( \beta_2,3 \) loop spectral density at \( \omega_{N} \) is detected for Rp-cAMPS relative to the other two bound states (Table 1 and Fig. S3a) supporting the conclusion that the Rp analog promotes a shift in the dynamics of the \( \beta_2,3 \) turn towards slower (> ~ns) time-scale motions consistently with the reported increase in the average \( J(0) \) and \( \Delta R_{2,eff} \) values.
4.3 Discussion

4.3.1 Current Model of cAMP-Dependent Allostery in PKA.

The understanding of the molecular basis underlying the different functions of the Sp-cAMPS agonist and the Rp-cAMPS antagonist requires that our results be interpreted in the context of the current model for the cAMP-dependent allosteric control of PKA (29, 66), which is here briefly reviewed. The function of PKA is controlled by cAMP through an allosteric mechanism in which the cAMP signal propagates beyond the immediate PBC boundaries through multiple intra-molecular signal pathways that cross the α/β-subdomain interface in CBD-A and ultimately perturb the R binding site for the catalytic subunit (C). Such pathways include both direct and indirect couplings between the PBC and the α-helical subdomain. Direct couplings involve a hydrophobic hinge cluster (12, 59) as well as a salt bridge between E200 and R241 (66) both connecting the PBC to the α-B/C helices. Indirect couplings are triggered through R209 in the PBC and are relayed by I163 (1) and D170 (29, 68), which are both interacting with R209 though hydrophobic and electrostatic interactions, respectively, as shown in Fig. 1f and in Fig. 7b.

4.3.2 An Effective NMR Signature for cAMP-Agonists and Antagonists.

Our NMR results reveal that overall the effect of Sp-cAMPS on R(CBD-A) resembles that of cAMP, while Rp-cAMPS displays a different behavior from cAMP, both in the terms of compounded chemical shift variations and of dynamic profile.
Specifically, a very distinct difference between Sp- and Rp-cAMPS appears by considering their effects on the three allosteric hot spots previously identified in the model of the cAMP-dependent allostery (i.e. I163, D170 and the $\alpha$-B/C boundary) (29). The comparative analysis of the compounded ppm changes reveals that Sp-cAMPS perturbs these three allosteric sites according to a pattern very similar to that observed for cAMP. Unlike Sp-cAMPS, the effect of Rp-cAMPS on the same three sites is markedly different from that of cAMP: the D170 region remains essentially unaffected by the binding of Rp-cAMPS to free RI$\alpha$ (119-244) and the other two loci display chemical shifts that are different from those of both the cAMP-bound and the free forms of CBD-A. In other words, the diverse ppm trends observed for Rp- and
Sp-cAMPS (Fig. 2) at the allosteric triad sites (i.e. I163, D170 and the α-B/C interface) define a clear NMR signature for the rapid identification of phosphorothioate cAMP antagonists and agonists (Fig. 2, red dotted rectangles). While this result will facilitate the discovery of future phosphorothioate cAMP antagonists though NMR-screening of cAMP-analog libraries, further improvements in the targeted design of cAMP analogs requires also that the functional differences between the Rp- and Sp-analogs be rationalized in terms of a molecular model. Such model should account not only for the chain of perturbations revealed by the ppm variations but also for the dynamical and thermodynamical (28) differences observed between the Rp- and Sp-cAMPS bound states of CBD-A.

4.3.3 A Molecular Model for the Rp-cAMPS Antagonism.

The chemical structures of cAMP and Rp-cAMPS differ uniquely for the replacement of the exocyclic equatorial oxygen of cAMP with a sulfur atom (Fig. 1b). While this might appear as a possibly minor isolobal substitution, the introduction of sulfur not only induces local chirality at the phosphorus stereocenter but also results in steric and electronic effects. The former are primarily caused by the larger van der Waals radius of sulfur relative to oxygen and the accordingly longer P-S bond, while the latter are due to the reduced resonance and the increased localization on the sulfur of the phosphorothioate charge (18, 65). Furthermore, the multiple interactions that involve the equatorial exocyclic position (with G199, E200 and R209 in Fig. 1f and Fig. 7b) relay and amplify the steric and electronic perturbations linked to the oxygen/sulfur replacement. Specifically, the
phosphorothioate negative charge primarily localized on the equatorial sulfur atom of Rp-cAMPS forms a very stable salt bridge with the guanidinium of R209 (27) and

Table 1. Relaxation Data Statistics for the $\beta_2-\beta_3$ Loop and the PBC.

<table>
<thead>
<tr>
<th></th>
<th>$\beta_2-\beta_3$ Loop [163-171]</th>
<th>PBC [199-211]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_p$-cAMPS</td>
<td>cAMP</td>
</tr>
<tr>
<td>$J(0) \times 10^9$ (s/rad)</td>
<td>3.72 ± 0.11</td>
<td>2.76 ± 0.05</td>
</tr>
<tr>
<td>$J(\omega_N) \times 10^{10}$ (s/rad)</td>
<td>2.27 ± 0.09</td>
<td>2.61 ± 0.05</td>
</tr>
<tr>
<td>$J(\omega_N+\omega_H) \times 10^{11}$ (s/rad)</td>
<td>0.45 ± 0.15</td>
<td>0.42 ± 0.08</td>
</tr>
<tr>
<td>$\Delta R_{2eff}$ (s$^{-1}$)</td>
<td>1.87 ± 0.46</td>
<td>0.13 ± 0.15</td>
</tr>
</tbody>
</table>

*Only residues for which data is available for all three ligands were considered in the computations of the averages, in order to avoid introducing biases into comparisons between different ligands.

competes with the carboxylate of D170 for the neutralization of the guanidinium positive charge. As a result, the anchoring of the D170 side chain to R209 is weakened in the Rp-cAMPS bound form as compared to the cAMP-bound state (18), as also confirmed by the compounded ppm changes suggesting that the D170 region is affected only by cAMP and Sp-cAMPS binding but not by Rp-cAMPS (Fig. 2). In other words, the D170 site behaves similarly in the Rp-cAMPS-bound form and in the free-state of CBD-A, which is able to generate a stable R:C complex. The weakened R209 anchoring of D170 in the Rp-cAMPS-bound form is also mimicked by the D170A mutation of the R-subunit (65). This mutant is non-allosteric because the cross-talk between the cAMP- and the C-binding sites of CBD-A is compromised by the D170A mutation, but the affinities for both binding partners are preserved.
leading to the formation of a stable cAMP:R(D170A):C ternary complex (65). These similarities between the Rp-cAMPS:R, free R and cAMP:R(D170A) point to the existence of a stable Rp-cAMPS:R:C ternary complex in which C is still inhibited even after Rp-cAMPS binding (Fig. 7b), explaining why Rp-cAMPS functions as a cAMP antagonist. In summary, the polarizing electronic effects of Rp-cAMPS on the guanidinium of R209 cause the obligatory intra-molecular cAMP signaling pathway relayed by D170 to be switched off, thus breaking the negative-cooperativity between the cAMP- and C-binding sites and stabilizing the ternary inhibitory intermediate.

The similarity between the Rp-cAMPS:R and free-states of R, and consequently the stability of the ternary Rp-cAMPS:R:C kinase inhibitory complex, is further supported by the similar Gibbs free energies of unfolding measured for these two forms of R through urea denaturation (28). The ΔG\text{unfolding} of cAMP-bound R-subunit decreases by more than 2 kcal/mol upon removal of cAMP and addition of Rp-cAMPS to the free form does not cause any significant ΔG\text{unfolding} change (28). This observation is fully consistent with the weakening of the R209/D170 salt bridge upon Rp-cAMPS binding and with the effect of Rp-cAMPS on the I163 site as revealed by our ppm data (Fig. 2d). Unlike D170, I163 is predominantly affected by steric effects mediated by the adjacent R209 methylenes. The Rp-cAMPS induced change in I163 represents a significant perturbation for CBD-A because I163 belongs to a core group of buried hydrophobic amino acids that are well shielded from the solvent and are likely a key determinant of the global unfolding of CBD-A. The
binding of Rp-cAMPS causes therefore a partial global unfolding of CBD-A which
enhances its conformational plasticity similarly to the free-state of CBD-A (28, 69,
70). The ensuing enhanced flexibility of the Rp-cAMPS-bound state of R relative to
its cAMP-bound form assists the α-subdomain structural rearrangement required to
recognize the C-subunit (20) and promotes the transition from the binary Rp-
cAMPS:R to the ternary Rp-cAMPS:R:C complex.

The formation of the ternary Rp-cAMPS:R:C complex is promoted not only
by the global unfolding modes triggered through the I163 pathway, but also by the
less global (ms-μs) motions that are specifically enhanced by Rp-cAMPS and not by
Sp-cAMPS. These Rp-cAMPS specific motions are confined to the PBC and to the
β2-β3 loop (Fig. 7b). The Rp-analog enhanced ms-μs dynamics in the PBC is
consistent with the extensive network of PBC interactions stemming from the
equatorial exocyclic position of cAMP, which involves not only R209 but also other
critical residues such as A202 at the N-terminus of the PBC α-B' helix (Fig. 1f). The
dynamics at the β2-β3 is explained by the Rp-cAMPS induced perturbations at I163
and D170 located at the N-and C-termini of this region, respectively. Furthermore,
the backbone CO and NH of R209 hydrogen bond the NH of G166 and the CO of
D167 (18), respectively, further propagating the equatorial sulfur induced effects
from the PBC to the β2-β3 loop. Both the PBC and the β2-β3 regions include sites
critical for C-binding (20) either directly through R:C contacts (i.e. the α-B' helix) or
indirectly through binding-coupled conformational changes (i.e. D170). It is
therefore possible that the enhanced ms-μs dynamics observed for the PBC and the β₂-β₃ loci of the Rp-cAMPS-bound state of R assist the initial recognition of the C-subunit by the Rp-cAMPS:R binary complex facilitating the formation of the ternary system that inhibits the kinase function.

\[ X+R:C \leftrightarrow [X:R:C] \leftrightarrow X:R+C \]

with \( X = \text{cAMP or Sp-cAMPS} \)

\[ X+R:C \leftrightarrow [X:R:C] \leftrightarrow X:R+C \]

with \( X = \text{Rp-cAMPS} \)

Figure 7. Proposed schematic model for the agonism (a) and the antagonism (b) of the Sp-cAMPS and Rp-cAMPS phosphorothioate cAMP-analogs. The structures of Sp-cAMPS and Rp-cAMPS bound R1α (119-244) are shown in panels (a) and (b), respectively, based on the PDB coordinates 1NE6 and 1NE4 (27). The bond and atom color coding is as in Fig. 1 (e,f). Residues that play critical roles in the model (i.e. R209, 1163 and D 170) are highlighted in bold. Selected secondary structure elements are indicated. The backbone ribbon is colored orange for regions that are significantly more dynamic in the ms-μs time-scale relative to the cAMP-bound state and grey otherwise. The Molmol program was used to create this Figure (58). Three state equilibria between the R:X and the R:C forms through the ternary X:R:C intermediate are also schematically shown in the bottom of panels (a) and (b). X denotes cAMP or one of its phosphorothioate analogs. R and C indicate the regulatory and catalytic subunits of PKA, respectively.
It should be noted that the model proposed here focuses on CBD-A, whereas the full length R-subunit is composed of two CBDs (CBD-A and CBD-B) (71), which communicate with each other allosterically. It is therefore possible that Rp-cAMPS not only affect the internal allostery of CBD-A as discussed here, but it could also interfere with the allosteric cross-talk between CBD-A and CBD-B, as previously suggested (27). The effect of Rp-cAMPS on CBD-A remains however critical to understand how this analog is able to competitively inhibit PKA, as already pointed out (26, 72).

4.3.4 *A Molecular Model for the Sp-cAMPS Agonism.*

As a result of the different local chirality between Rp- and Sp-cAMPS, the interaction networks propagating from the sulfur atoms of the Rp- and Sp-analogs are markedly different (Fig. 1e, f and Fig. 7a, b). Unlike Rp-cAMPS, the axial sulfur atom of Sp-cAMPS does not interact with R209 and therefore it does not affect the two key allosteric sites linked to it (*i.e.* D170 and I163) differently from cAMP. The Sp-cAMPS steric and electronic effects are mainly localized on A210 (Fig. 2c). The only long-range perturbation caused by the cAMP/Sp-cAMPS replacement is observed for N85 (Fig. 2c), which is caused by a steric effect on A210 relayed by Q165 to the proximal N185. This residue is located in the loop between strands $\beta_4$ and $\beta_5$, a region characterized by the highest sequence variability among different cAMP-binding domains (73) and not involved in the binding of cAMP or C. The perturbations caused by the sulfur-to-oxygen replacement at the axial exocyclic position are therefore not functionally relevant and explain the similar allosteric
profile observed for cAMP and Sp-cAMPS, rationalizing the Sp-cAMPS agonism. Consistently with the agonist function of Sp-cAMPS, it is anticipated that the ternary Sp-cAMPS:R:C intermediate (Fig. 7a) is significantly less stable than the Rp-cAMPS:R:C inhibitory complex (Fig. 7b). It should however be noted that the ternary intermediate could still be formed, at least transiently and at low concentrations, even in the case of cAMP and of the functionally homologous Sp-cAMPS (Fig. 7a). This hypothesis is supported by the dynamics of the cAMP-bound state of CBD-A, as explained in the following section, as well as by the recent finding that cAMP still binds the PKA holoenzyme with a µM affinity (74).

4.3.5 Functional Relevance of the Residual Dynamics in the cAMP-Bound State.

The functional relevance of the CBD-A dynamics in its cAMP-bound state is evaluated in relation to the R:C binding sites. The recently solved structure of the R(CBD-A):C complex (20) has revealed three key R(CBD-A):C interactions sites that map to the α-B’ helix, the C-helix and the α-Xn/A-helix turn (Fig. 5, black dashed contours). All three R:C interaction regions within CBD-A match surprisingly well with the dynamic “hot-spots” observed for the cAMP-bound state of RIA (119-244) (Fig. 5a-d). The α-Xn/A-helix loop and the PBC are two of the most significant clusters subject to ms-µs motions (Figs. 3b,d,e, 4a and 5c,d), while the C-helix is intrinsically dynamic and highly conformationally heterogeneous in the ps-ns time-scale (Figs. 3a-c, 4a-c, 5a, b and 6), in full agreement with recent molecular dynamics simulations (66, 75). The structure of the R(CBD-A):C complex (20) has also revealed that significant local conformational differences exists between
the C- and cAMP-bound states of CBD-A for loci not involved in direct interactions with C. These sites of local conformational divergence between the cAMP- and the C-bound states of the CBD-A are localized in the vicinity of D170 and at the C-terminus of the α-X₅-helix (Fig. 5, red dashed contours) and correlate well with those that are dynamic in the ms-μs time-scale (Fig. 5c,d) and, in the case of D170, in the ps-ns time-frame as well (Fig. 5a, b).

The residual dynamics of the cAMP-bound state of CBD-A may reflect at least in part its intrinsic flexibility as is probably the case for the loop region between the β-stands 4 and 5, which is known to be poorly structurally defined in several CBDs of R-isoforms (73). However, the overall correlation observed between the dynamic hot-spots of R1α (119-244) and the sites of C-binding and C-binding induced conformation changes (Fig. 5) suggests (76) that the residual dynamics observed for the cAMP-bound state of CBD-A may assist the early recognition of the C-subunit even before cAMP is released from the R-subunit, leading to the transient formation of the cAMP:R:C ternary intermediate as hypothesized above. Consistently with this hypothesis, the residual dynamic hot-spots identified for the cAMP-bound CBD-A may facilitate the reversible toggling of the R-subunit between the cAMP-bound and the C-bound states, namely, the reversible transition from the active to the inactive state of protein kinase A. Considering that the preservation of ms-μs dynamics even after ligand binding has been recently reported also for other systems (77, 78), it is possible that the residual plasticity of the ligand-bound state
represents a general signaling mechanism that has evolved to ensure the reversibility in the binding to mutually exclusive partners.

4.4 Conclusions

We have mapped by $N_z$-exchange NMR spectroscopy and $^{15}$N relaxation measurements the interactions and the dynamics of the binary R(CBD-A):Sp-cAMPS and R(CBD-A):Rp-cAMPS complexes. The comparative analysis of these phosphorothioate-bound forms has revealed significant allosteric and dynamic differences between the Rp-cAMPS- and cAMP-bound states, whereas the Sp-cAMPS-bound form behaves similarly to the cAMP:R(CBD-A) complex, in agreement with the functional diversity between the Rp- and Sp-analogs. These data have led to the proposal of a model for the Rp-/Sp-cAMPS antagonism and agonism based on steric and electronic effects on two key allosteric relay sites I163 and D170. The perturbations caused by Rp-cAMPS at these sites not only result in increased ms-μs flexibility for the local motions confined to the PBC and to the proximal $\beta_2-\beta_3$ loop, but they also enhance global partial unfolding modes. Both effects promote the conformational plasticity at the $\alpha/\beta$-subdomain interface and therefore the stabilization of a ternary inhibitory Rp-cAMPS:R:C complex. In the presence of Sp-cAMPS or cAMP, which do not affect the I163 and D170 allosteric sites as Rp-cAMPS, this ternary intermediate is less stable but it can still form at least transiently and with lower populations as suggested by the correlation between several
sites of residual ms-μs and ps-ns dynamics in the cAMP- and Sp-cAMPS-bound states of R(CBD-A) and the loci of C-binding and of C-induced local conformational change. Such correlation points to an ancillary role of the residual cAMP-bound state dynamics to assist the early recognition of free C by R even before cAMP dissociates from R, supporting the transient existence of the ternary complex involving cAMP, R and C and facilitating the transition from the ‘on’ to the ‘off’ state of PKA. These considerations suggest a general signaling mechanism adopted and conserved by proteins with mutually exclusive binding states to ensure reversibility in the interactions with their molecular partners.

In summary, the proposed model rationalizes the functional, dynamical and thermodynamical diversity of the Rp- and Sp-cAMPS analogs and refines our understanding of allostery in CBDs. In addition, we expect that the differential NMR patterns established here for Rp-cAMPS and Sp-cAMPS may serve as an NMR signature for the discovery of novel phosphorothioate-based cAMP-agonists and antagonists through the rapid screening of ligand libraries. The leads thus discovered will be further improved by rational design based on the proposed model of cAMP-agonism and antagonism.

4.5 Acknowledgements: We thank Dr. Susan S. Taylor for generously providing the RI clone (119-244) and together with Dr. Mona Abu-Abed for insightful discussions. We are also grateful to the Canadian Institutes of Health Research (CIHR) for
financial support and to the Heart and Stroke Foundation of Canada for a Maureen Andrew New Investigator award to GM.

4.6 Foot Notes: Supplementary information includes Additional Tables and Figures with distances, hydrodynamics, relaxation analyses is presented in the supplementary material. Model-free analysis of cAMP: R1α (119-244) is described in appendix.

4.7 References


4.8 Supplementary Material

4.8.1 NMR Sample Preparation of RIα (119 – 244) Bound to Different Ligands.

In the analysis of the $^{15}$N R$_1$, R$_2$ and NOE data one of the primary considerations is the overall flexibility of the α-subdomain of CBD-A, as previously shown by H/D exchange experiments monitored by mass spectrometry (30, 59). Conformational changes in the α-subdomain have the potential to affect the diffusional model describing the overall tumbling of the protein in solution. For instance, hydrodynamic simulations show that the re-orientation of the α-helices occurring in CBD-A upon C-subunit binding causes a dramatic shift from an oblate to a prolate model (Supplementary Material). This examples illustrates how the conformational plasticity of the α-subdomain of CBD-A hampers the accurate description of the overall motion of RIα (119-244) in terms of a single well-defined diffusion tensor. As a result the analysis of the $^{15}$N relaxation rates and NOEs of this construct in terms of the Lipari-Szabo Model Free parameters using a single diffusion
tensor (60, 61) may not be adequate. This is why we relied on the reduced spectral
density mapping approach, which does not require assumptions on the overall
tumbling of the protein or on the exact form of the spectral density functions (52-55).

The residue-dependent variations in the spectral density values likely reflect
not only the heterogeneity in the internal motions of R1α (119-244), but also to some
extent the anisotropic nature of its overall tumbling. This is confirmed by the non-
negligible deviations from one of the D_{par}/D_{per} ratios computed for the available
structures of CBD-A (Supplementary Material). In order to assess the possible
contributions from the anisotropy of the overall motions to the experimental
spectral densities and thus obtain a better insight on the internal motions, the
experimental spectral density values were compared to those predicted through rigid
bead model hydrodynamic simulations (56, 57). These computations are based on
representative reference conformations of R1α (119-244). Such CBD-A structures
include not only that of the cAMP-bound form, but also that of the C-bound state
(Supplementary Material) which is here used simply as a representative extreme
element of a drastic conformational rearrangement within the α-subdomain. In
addition, regions subject to ms-μs dynamics were further characterized by the
analysis of the R R_{2} products, which are effectively insensitive to variations caused
by diffusional anisotropy as previously shown (62) and as also confirmed in our
specific case by hydrodynamic modeling (Supplementary Material). Furthermore,
ms motions were independently probed by relaxation-compensated constant-time
CPMG experiments, as described above.
**Figure S1.** Evaluation of the effect of a shift from an oblate to a prolate diffusional anisotropy model on the $^{15}$N relaxation rates predicted through hydrodynamic modeling. As in Fig. 3 the red continuous lines indicate the $^{15}$N relaxation rates computed by HYDONMR for the cAMP-bound state (PDB code 1RGS; oblate diffusion model), while in green are shown the corresponding rates similarly predicted for CBD-A in its C-bound conformation (PDB code 1U7E in which the two C-terminal residues 243 and 244 are not available likely because too flexible; prolate diffusion model). The meaning of thick and thin lines is as explained in the caption of Fig. 3. For reference purposes only the experimental data for the cAMP-bound state (black) is shown. The data for the Rp- and Sp-cAMPS analogs is not displayed to avoid crowding of the panels.
Figure S2. Evaluation of the effect of a shift from an oblate to a prolate diffusional anisotropy model on the reduced spectral densities predicted through hydrodynamic modeling. The color coding is as in Fig. S1.
Figure S3. 2D reduced spectral density plots of $J(\omega)$ vs. $J(0)$ for the $\beta_2-\beta_3$ loop (a) and for the PBC (b) regions of R1\alpha (119-244) bound to Sp-cAMPS (green) and to Rp-cAMPS (orange). The solid red line is derived from the theoretical spectral densities computed using a Lorentzian and assuming isotropic diffusion of a rigid rotor.
Table S1. Distances between Selected Atoms in the Crystal Structures of Sp-cAMPS$, cAMP^b$ and Rp-cAMPS$^c$ Bound R1α.

<table>
<thead>
<tr>
<th>Residues</th>
<th>Atoms</th>
<th>Sp-cAMPS (Å)</th>
<th>cAMP (Å)</th>
<th>Rp-cAMPS (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP(S) – A210</td>
<td>O2P/S2P – NH</td>
<td>3.36</td>
<td>2.87</td>
<td>2.84</td>
</tr>
<tr>
<td>Q165 – A210</td>
<td>CG – C</td>
<td>3.67</td>
<td>4.36</td>
<td>3.88</td>
</tr>
<tr>
<td></td>
<td>CG – N</td>
<td>4.76</td>
<td>5.18</td>
<td>4.86</td>
</tr>
<tr>
<td></td>
<td>OE1 – O</td>
<td>3.86</td>
<td>4.7</td>
<td>4.14</td>
</tr>
</tbody>
</table>

$^a$ PDB code: 1NE6; $^b$ PDB code: 1RGS; $^c$ PDB code: 1NE4.

Table S2. Overall Tumbling Parameters Computed through Hydrodynamic Simulations for Different States of R1α (112-244).$^1$

<table>
<thead>
<tr>
<th>CBD-A Bound State</th>
<th>PDB Structure</th>
<th>$&lt;\tau_m&gt;$ (ns)$^b$</th>
<th>$D_{par}/D_{per}$ $^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP</td>
<td>1RGS</td>
<td>7.0 – 7.6</td>
<td>0.81</td>
</tr>
<tr>
<td>Sp-cAMPS</td>
<td>1NE6</td>
<td>7.2 – 7.4</td>
<td>0.81</td>
</tr>
<tr>
<td>Rp-cAMPS</td>
<td>1NE4</td>
<td>7.1 – 7.5</td>
<td>0.81</td>
</tr>
<tr>
<td>C-Subunit$^e$</td>
<td>1U7E</td>
<td>7.0 – 7.6</td>
<td>1.59</td>
</tr>
</tbody>
</table>

$^a$ For all computations the HYDRONMR program (56, 57) was used as explained in the Materials and Methods section. $^b$ Range of harmonic mean overall tumbling correlation times calculated with an atomic element radius of 3.3 Å ± 0.2 Å. $^c$ Only the R1α residues of the PDB 1UE7 structure were employed for this computation. See also caption of Fig. S1. $^d$ No significant dependence of the $D_{par}/D_{per}$ ratio on the atomic element radius in the 3.3 Å ± 0.2 Å range was observed.
EPAC ACTIVATION AS REVEALED BY THE SOLUTION STRUCTURE OF THE HOLO EPAC1 CBD

CHAPTER 5
Chapter Two Preference

Assignment of *apo*-EPAC1 (149-305) and EPAC1 (149-318) in cAMP free and bound states were completed by Mohammad Taghi Mazhab Jafari in the Melacini laboratory.
EPAC ACTIVATION AS REVEALED BY THE SOLUTION STRUCTURE OF THE HOLO EPAC1 CBD

The structure of EPAC2 in its auto-inhibited cAMP free state reveals an overall closed conformation in which the regulatory region covers the CDC25HD domain and sterically hinders access of Rap proteins to the catalytic region (65). This structure has also unveiled that a salt bridge cluster ('ionic latch') between the catalytic domain and the N-terminal helical bundle (NTHB) of the CBD is key for this inhibition (see Fig. 9c,d; Chapter 1) (65). Biochemical characterizations including mutational analyses suggest that EPAC is allosterically activated upon cAMP binding to its CBD, which consequently removes the steric hindrance by displacing the regulatory region away from the catalytic region (9). While the proposed model however at present is only hypothetical, it clearly underlines the importance of CBD as the 'central controlling unit' of the EPAC activity.

![Figure 1. Schematic representation of the primary structure of EPAC1 (a) and of the EPAC1h (149-305) construct used for the NMR studies presented here (b).](image-url)
In this chapter a mechanism for the cAMP-dependent activation of EPAC has been proposed based on the solution structure of cAMP-bound EPAC₁₉(149-305) (Fig. 1) as well as on the comparative NMR analysis of the apo and holo state of the same construct. The EPAC₁₉ (149-305) construct comprises the CBD regions that were previously shown to be critical for the cAMP-dependent activation of EPAC (i.e. the ionic latch, the PBC and the C-terminal hinge helix) (9, 67). EPAC₁₉ (149-305) does not include the lid, which contains H317, a residue suggested to stack against the adenine base of the cAMP. However, mutation of H317 to alanine and molecular dynamics simulation showed that the lid does not alter the cAMP sensitivity or dependence (69, 110). Therefore EPAC₁₉ (149-305) is a suitable construct to start investigating the cAMP-dependent activation of the EPAC CBD. Our earlier attempts to comparatively analyze the apo and holo CBDs of PKA were hampered by the instability of the PKA apo state. The stability of apo and holo EPAC1 provides therefore a unique opportunity to study the cAMP-dependent allosteric network of eukaryotic CBD at residue resolution by NMR. The results reported in this chapter not only confirm the previously hypothesized allosteric pathway relayed through the hinge region but also propose a more extended long-range cAMP-dependent allosteric signaling mechanism. Moreover, structural comparisons between the PKA and EPAC CBDs reveal some remarkable differences that were not anticipated before.
5.1 **Materials and Methods**

5.1.1 *Protein expression and purification*

The cDNA for EPAC1_h (149-305) was prepared by inserting two stop codons in the EPAC1_h (149-881) DNA cloned into the pGEX-4T3 vector (kindly made available to us by Dr. X. D. Cheng UTMB). The protein was expressed as a GST-fusion construct under the control of the *lac* promoter. The fusion protein was expressed and purified according to the previously established protocols (11, 67). In brief, the plasmid containing the EPAC1_h (149-305) DNA was transformed into an *E. coli* BL21(DE3) codon plus strain and used for over-expressing. $^{15}$N/$^{13}$C single labeled or double labeled proteins were over expressed by fermentation in a M9 minimal media supplemented with trace metals, vitamins (10 µg/L thiamine and biotin), salts (NaCl, KH$_2$PO$_4$, and Na$_2$HPO$_4$), 1 g/L $^{15}$N-ammonium chloride, 100 µg/mL ampicillin, and 3 g/L $^{12/13}$C-glucose for single or double labeled protein samples, respectively. The cultures were grown at 37 °C until an O.D$_{600}$ of 0.6, followed by induction with 1 mM IPTG for 3 h. at 37 °C. Cells were harvested by centrifugation for 5 min at 9000 ×g and stored at -80 °C. In order to purify the over expressed fusion protein the pellets were re-suspended in lysis buffer (PBS, 10 mM EDTA, 10 % v/v glycerol) supplemented with protease inhibitors and lysed by passage through an EmulsiFlex-C5 homogenizer (AVESTIN) at 5000 PSI. The cell debris was removed by centrifugation at 20,000 ×g for at least 45 min. The supernatant containing the GST-EPAC1_h (149-
305) was incubated with pre-equilibrated Glutathione Sepharose4 Fast Flow beads (Amersham Biosciences, Inc.) for 4 hours. The beads were extensively washed with 0.5 M NaCl and the EPAC1h (149-305) protein was cleaved from the GST tag using biotinylated thrombin. Thrombin was subsequently removed by incubating with Streptavidin beads and the protein was further purified through a Q-column (GE Health Sciences). The pure protein was then dialyzed against the low salt NMR buffer (50 mM HEPES at pH 7.6, 50 mM NaCl, 1 mM TCEP, 0.02% w/v NaN₃). For preparation of the NMR samples, the protein was then concentrated to 0.1 - 0.5 mM after adding 1 mM TCEP, 2 mM cAMP (if necessary) and 5 % v/v D₂O.

5.1.2 NMR spectroscopy

All NMR experiments were performed at 306 K on a Bruker AV spectrometer fitted with a TCI cryo-probe at a ¹H frequency of 700 MHz. The temperature of the spectrometer was calibrated with both a thermocouple and an ethylene glycol sample. The 2D-HSQC spectra were acquired with spectral widths of 31.8 and 14.2 ppm for the ¹⁵N and ¹H dimensions, respectively and with 128 (¹⁵N) and 1024 (¹H) complex points. The carrier frequencies of the proton and nitrogen channels were centered on 4.7 ppm and 119 ppm, respectively. The chemical shifts for the protons were referenced relative to DSS and the nitrogen dimension was then indirectly referenced using the nitrogen to proton gyromagnetic ratio. ¹⁵N decoupling strength was set as described in chapter 2. All the NMR spectra were processed and analyzed as explained in Chapter 3 (114). The
assignments of the cAMP-bound of EPAC1h (149-305) were obtained through standard sets of triple resonance experiments (115): HNCO, HNCA, HN(CO)CA, CBCA(CO)N~3, HBHA(CO)NH, HNCACB and HNHAHB. The rest of the backbone and side chain assignment were obtained with $^{15}$N NOESY-HSQC, $^{15}$N HSQC-NOESY-$^{15}$N HSQC, $^{13}$C HSQC-NOESY-$^{15}$N HSQC and $^{13}$C HSQC-NOESY-$^{13}$C HSQC. The intermolecular NOE between cAMP and the protein for the ligand docking was determined through $^{13}$C edited NOESY acquired with 2048, 128 and 170 complex points in t1, t2 and t3 and with spectral widths of 9842, 13192 and 9842 Hz, respectively (116). All the NOE experiments were recorded with mixing time of 120ms. The secondary structure probabilities were obtained from the secondary chemical shifts using the PECAN program (117). Compounded chemical shift differences between the cAMP-bound and free states of EPAC1h (149-305) were calculated as explained in Chapter 3. The backbone assignment of EPAC1h (149-305) apo and EPAC1h(149-318) holo and apo was completed by Mohammad Taghi Mazhab Jafari in the Melacini lab.

5.1.2.1 H/D and H/H Exchange experiment

H/D exchange experiments were performed by diluting 0.25 mM U-$^{15}$N EPAC1h (149-305) into 99.9 % D$_2$O buffer (50 mM HEPES, 50 mM NaCl, 1 mM TCEP, and 0.02 % w/v NaN$_3$). For H/H exchange experiments a 0.2 mM sample was prepared in NMR buffer in presence of 2mM cAMP. The rest of the experimental conditions and data analysis including the rates for H/D and H/H exchange were determined as described previously in Chapter 3.
5.1.2.2 Residual dipolar coupling measurements

Residual dipolar coupling for the backbone amide vectors were calculated at 306 K from the difference between the scalar couplings measured in the presence and absence of pf1 (from Alsa Biotech) using a sensitivity-enhanced IPAP-type $^1$H, $^{15}$N HSQC experiment (118). The anisotopic sample of EPAC1_h (149-305) was prepared by mixing a 0.2 mM protein solution in the NMR buffer with 17 mg/ml of pf1 phages. The NMR data was processed and analyzed as before. The RDC data analysis was performed using the program Pales (119), by fitting the experimental RDC data to several crystal structures (i.e. EPAC2, regulatory subunit RIα of PKA B domain, CNG and HCN from the PDB files 1O7F, 1RGi3, 1RL3, 1VP6 and 1U12, respectively) (119). The degree of agreement between the experimental data and the crystal structure was determined from the $\chi^2$ test, the RMSD values, the correlation coefficients (R) and the quality factors (Q). The residual dipolar coupling data was also used in the refinement of the EPAC1_h(149-305) solution structure.

5.1.2.3 Determination of the Adenine Base Orientation in EPAC-Bound cAMP

The double labeled $^{13}$C/$^{15}$N protein and cAMP samples were prepared in a D$_2$O solution composed of 20 mM phosphate buffer at pH 7.5 and 50 mM NaCl. The free cAMP was assigned with 2D homonuclear ($^1$H-$^1$H) TOCSY spectra measured with 2048 and 512 complex points and spectral widths of 8389 and 8389 Hz, respectively. The TOCSY spinlock was set at 60 ms with 10 kHz strength. The conformation of free cAMP was probed through an off-resonance
ROESY (ORR) experiment (120). In the ORR experiments the mixing time was set at 158 ms and $t_1$, $t_2$ at 2048, 700 complex points respectively, with a spectral width of 8389 Hz for both dimensions. Cross-peaks arising from cross-relaxation and chemical-exchange were distinguished based on their sign: cross-relaxation peaks are of opposite sign from the diagonal, whereas peaks due to chemical exchange are of the same sign as diagonal. The orientation of the adenine base (i.e. syn or anti) in EPAC bound cAMP was determined from the transfer NOEs observed in a 2D $^{13}$C/$^{15}$N double filtered edited NOESY experiment measured with 2048 and 420 complex points and spectral widths of 8389 and 8389 Hz, respectively (116). The sample was prepared by concentrating the double labeled protein to 0.3 mM in the presence of 0.7 mM cAMP in D$_2$O buffer. The NOE mixing time was set at 80 ms. The cross-relaxation peaks were differentiated from the exchange peaks by a 2D-ROESY experiment recorded at a spectral width of 11 ppm in both dimensions and with 1K and 200 complex points, respectively. The mixing time was set at 15 ms and the CW spin-lock strength at 2.5 kHz.

5.1.2.4 Structure Calculations

The structure of cAMP-bound EPAC1_h (149-305) was calculated using the program CNS (1.2 version) (121). The topology and the parameter files for cAMP hetero-atoms were generated from the Dundee PRODRG2 server (122). At this stage care must be taken to name the ligand in the same way as in the ligand input file (ligand.seq) and the topology file (line begins with RESidue). The $\mathrm{C}_\alpha$, $\mathrm{C}_\beta$ and $\mathrm{H}_\alpha$ chemical shifts were used to determine the regions of regular secondary
structure. The dihedral restraint file was obtained from the comparison of the PECAN based secondary structure and crystal structures of apo-EPAC2 (PDB 1O7F). Dihedral angles for the well defined secondary regions were included based on 75\% agreement between the NMR based secondary structure and crystal structure. Proton – proton distance restraints were obtained from the peak intensities of the NOESY experiments. The highest intensity peaks (strong NOE) were assigned a distance restraint of 2.5 Å (+) 0.7 (-) 0.3, the medium NOEs and the weak NOEs were assigned a distance restraint of 3.0 Å (+) 1.2 (-) 0.3 and 4.0 Å (+) 2.2 (-) 1.0, respectively. In addition, hydrogen bond restraints were also introduced as restraints for those residues showing protection from H-D exchange. Two distance restraints were defined for each hydrogen bond: 1.8 – 2.0 Å for the HN-O distance and 2.7 – 3.0 Å for the N-O distance (123). The structure was further refined with NH RDC restrain calculations. In order to facilitate faster structure calculations, the N-terminal unstructured part (residues 149-166) were not included in the final structure calculations. First, the parameter file (file name: protein.mtf) for the protein was generated using the protein sequence (file name: protein.seq) and ligand (file name: ligand.seq) and their respective topology and parameter files. Subsequently the EPAC structure was generated in a completely extended conformation using the protein parameter file. In order to implement the RDC constraints the extended PDB was modified by adding the following lines after the last residue:

ATOM  2277  X  ANI  500  475.575  2.33  -8.214  1.00  0.000
The protein.mtf file was also modified accordingly by adding following lines:

2277 '500' 'ANI' 'X' 'XXX'  0.00000E+00  10.0000
2278 '500' 'ANI' 'Y' 'YYY'  0.00000E+00  10.0000
2279 '500' 'ANI' 'Z' 'ZZZ'  0.00000E+00  10.0000
2280 '500' 'ANI' 'OO' 'OOO'  0.00000E+00  10.0000

And also:
2280 2277
2280 2278
2280 2279

And also
2277 2280 2278
2277 2280 2279
2278 2280 2279

Along with the protein and ligand parameter files the axis.param file was also included in the annealing protocol to implement the RDC restraints in the final refinement step. The RDCs were implemented using the Susceptibility ANIsotropy restraint (SANI) protocol of the CNS program 1.2 Version (121). The values for the SANI co-efficient DFS, axial and rhombicity components were calculated using the programs Pales (119) or DipoCoup (137).
The structure was calculated using standard simulated annealing protocols of CNS program (121). The protein and the cAMP molecules were allowed to anneal during a high temperature search phase of 0.015 ps molecular dynamic (MD) of 1000 steps. The temperature was lowered subsequently in two steps (3000 steps of MD of 0.015 ps in each step and 0.005 ps in each step, respectively). Finally 100 cycles of 4000 steps of Powell energy minimization were executed. The ensemble of 10 lowest energy structures showing zero violations of NOE and RDCs in the threshold range of 0.5 Å and 1.0 Hz, respectively, were selected. Ten low energy structures were further refined by implementing moderate temperature (500 K) restrained molecular dynamics and energy minimizations in an explicit shell of water (32). The quality of the structures was assessed using the program PROCHECK-NMR (124) and their RMSD deviation was calculated using Molmol (125). The final structures were visualized with the program Pymol (13) or Molmol.

5.2 Results and Discussion

5.2.1 Assignment of Holo-EPAC1_h (149-305)

The assignment for backbone HN, N, Cα, Hα, C' and side chains including Cβ, Hα and other residues was completed with triple resonance experiments as described in the material and method section. 3D experiments such as HNCO, HNCA, HN(CO)CA, CBCA(CO)NH, HBHA(CO)NH, HNCACB and
Figure 2. Strip plots for the 3D HNCA (a) and $^{15}$N HSQC NOESY $^{15}$N HSQC (b) of EPAC1$_b$ (149-305) demonstrating the sequential assignment for residues N301-K305 of EPAC1$_b$ (149-305). In both panels the auto peaks on the diagonal are indicated at the bottom of each spectrum.
HNHAHB were used to assign the HN(i), N (i), Cα(i), Hα(i), Cβ(i), Hβ(i), C’, Cα(i-1), Hα(i-1), Cβ(i-1) and Hβ(i-1) frequencies (115). The side chains were assigned using double and triple resonance NOESY experiments like $^{15}$N NOESY-HSQC, $^{15}$N HSQC-NOESY-$^{15}$N HSQC, $^{13}$C HSQC-NOESY-$^{15}$N HSQC and $^{13}$C HSQC-NOESY-$^{13}$C HSQC (116). Figure 2 shows the strip plot for a sequence of amino acid residues in EPAC1$_h$(149-305) that were assigned using the HNCA (Fig. 2a) and the $^{15}$N HSQC-NOESY-$^{15}$N HSQC (Fig. 2b) experiments. The 3D HNCA experiment provides sequential connectivity between the backbone HN(i), N (i), Cα(i), and Cα(i-1) frequencies of an amino acid residues in a polypeptide chain. Whereas, the HN-HN NOEs can provide information about adjacent amide protons in a helical stretch. In this particular instance the residues between 301-305 form a short helix, therefore the HN-HN NOEs will show a strong sequential NOE between residues i, i±1 and occasionally medium range i±2 NOEs for amide residues (Fig. 2b). Out of 156 amino acid residues of EPAC1$_h$ (149-305) five of which are proline, resonance assignments for 138 residues were obtained. In other word, ~91% of backbone resonances and ~70% of side chain resonances were assigned.

Initial secondary structure analyses with PECAN using the secondary chemical shifts of Hα, Cα, Cβ and C’ suggest that the solution structure of EPAC1$_h$ (149-305) preserves the general features typical of CBDs. Figure 3 shows the comparison of the holo-EPAC1$_h$ (149-305) secondary structure probabilities with those obtained from the crystal structure of apo-EPAC2 CBD-B.
Overall there is a good agreement between the two secondary structure profiles with two main exceptions: the \( \alpha_1 \) and \( \alpha_3 \) helices in the N-terminal helical bundle (NTHB).

**Figure 3. Chemical shift based secondary structure analysis of EPAC\( _{1h} \) (149-305).** The solid line indicate the secondary structure probability of EPAC1 based on secondary chemical shifts and dotted line represents the secondary structure of EPAC2 CBD based on the crystal structure (PDB 1O7F). The positive and negative value indicates probability of alfa helix and beta strand, respectively. The N-terminal helical bundle (NTHB), base binding region (BBR) and the phosphate binding cassette (PBC) are highlighted by blue, red and purple colour respectively.

The \( \alpha_1 \) helix may be destabilized due to absence of DEP domain, which in full length protein is connected to the CBD-B through the \( \alpha_1 \) helix. However it has been shown that deletion of the DEP domain does not affect the cAMP dependence activity of EPAC (38, 127). On the other hand the destabilization of \( \alpha_3 \) may reflect the intrinsic dynamic property of this region, which is also common to domain A of R1\( \alpha \) (Chapter 4). Therefore this construct of EPAC\( _{1h} \) provides a useful model system to evaluate the effect of cAMP-binding on EPAC.
Residual dipolar coupling of apo and holo EPAC1h(149-305)

In order to understand the cAMP dependent allostery in EPAC it is a key to characterize the solution conformation of the apo and holo (i.e. cAMP-bound) states of EPAC. Earlier, residual dipolar couplings (RDC) have been shown to be a useful tool in determining the domain orientation as well as the structural ensemble of proteins (128-130). In an isotropic solution the dipolar couplings are averaged to zero due to the macromolecular rotational tumbling. However, in an anisotropic medium, where macromolecules are partially aligned with respect to the magnetic field, the dipolar couplings are not fully averaged to zero resulting in detectable and informative coupling values called residual dipolar couplings (131, 132). The partial alignment of EPAC samples was achieved through weak electrostatic interactions with pfl phages (133). Our compounded chemical shift comparisons (Fig. 8) along with recently published secondary structural changes between apo and holo EPAC1h (134) showed that the cAMP-dependent changes are localized to four main ‘hot spots’: the N-terminal helical bundle (NTHB), β2-β3 loop, PBC and the hinge region. The NTHB and the β2-β3 loop regions were not studied by RDC since this region is very dynamic, we therefore analyzed the experimental RDC of the well structured β-barrel region, the PBC and the hinge helix by fitting them to the calculated RDCs from the crystal structures of apo or holo CBDs from PKA, EPAC2 and CNG (25, 57, 67). RDCs were measured for the apo or holo states of the EPAC1h (149-305) and EPAC1h (149-318) constructs. The EPAC1h (149-305) and EPAC1h (149-318) constructs differ only in the length
of the hinge helix and in the lid region, which was previously proposed to interact with the adenine base of cAMP and PBC based on comparisons between the EPAC and PKA systems (67).

Tables 1-4 and Figures 4 & 5 illustrate the fitting between the experimental RDCs (measured with and without cAMP) for the EPAC1_h(149-305) and EPAC1_h(149-318) constructs and the calculated RDCs from the crystal structures of the apo or holo states of EPAC2 CBD-B, PKA RIα domain-B and CNG. The best fit structure was determined on the basis of the $\chi^2$, the RMSD, the correlation coefficient (R) and the quality factor $Q$ (132, 135). The Q factors are used to evaluate the quality of the NMR structure, a concept similar to the R values used in the crystallography. The quality factor commonly used to evaluate the agreement between the calculated dipolar couplings from a known structure ($D_{\text{calc}}$) and observed dipolar couplings ($D_{\text{obs}}$):

$$Q = \frac{\text{rms}(D_{\text{calc}} - D_{\text{obs}})}{\text{rms}(D_{\text{obs}})}$$

6.1

In general terms, a Q factor below 25% is associated with a very high quality structure (135), whereas a 2.5 Å structure will yield a Q factor of 40%. For example, Table 3 and Figure 5 c,f and i show that the RDC values for the hinge region of holo-EPAC1_h(149-318) fit to the PKA RIα-B domain with a low Q factor indicating a very well defined structure. However other structures such as EPAC2 show high Q values might suggest a distribution of alignment tensors due to conformational fluctuations of the hinge region. Overall our analysis indicates that the experimental RDCs for the β-sub-domains of the EPAC1_h (149-305) and
EPAC1h (149-318) constructs in the cAMP-bound or free states have similar fitting against most of the CBD structures, with higher Q factors (more than 25%) (Tables 1-4; Figs. 4 and 5). The experimental RDCs of the PBC in EPAC1h apo state fit to the apo crystal structure of PKA and EPAC2 CBDs, confirming that the apo-EPAC1h PBC forms a similar structure to that in full length apo-EPAC2. Likewise the hinge region of apo EPAC1h also orients to a similar conformation to that of the apo-EPAC2 structure. Contrary to our expectations, the cAMP-bound EPAC1h RDCs for the PBC point to a conformation similar to that of the apo EPAC2 or hclo PKA crystal structures but with high Q factor (Tables 1 and 3; Figs. 4 and 5). The RDC data analysis for the hinge region in the presence of cAMP, shows a good agreement between the experimental RDCs and the predicted RDCs obtained from the PKA R1α-B cAMP bound structure (Table 1 and 3; Figs. 4 and 5). In other words the RDC analysis suggests that cAMP binding will not induce any conformational change in the β-barrel region or PBC of EPAC1h. However, the high Q values indicate that the binding of cAMP will increase the rate of conformational fluctuation between open and closed state of PBC. Based on the RDC analysis, the most significant changes are predicted in the hinge region structure (57).
Table 1. Fits of cAMP bound EPAC1b (149-305) RDCs.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\chi^2$</th>
<th>RMSD (Hz)</th>
<th>R</th>
<th>$Q^c$</th>
<th>N $d$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta^e$</td>
<td>PBC$^g$</td>
<td>Hinge$^h$</td>
<td>$\beta^e$</td>
<td>PBC$^g$</td>
</tr>
<tr>
<td>EPAC2$f$</td>
<td>124</td>
<td>66</td>
<td>93.8</td>
<td>3.52</td>
<td>2.89</td>
</tr>
<tr>
<td>Rl$\beta_b$</td>
<td>156</td>
<td>48</td>
<td>12.7</td>
<td>4.17</td>
<td>2.47</td>
</tr>
<tr>
<td>Rl$\beta_f$</td>
<td>220</td>
<td>440</td>
<td>209</td>
<td>4.95</td>
<td>7.42</td>
</tr>
<tr>
<td>CNG$h_b$</td>
<td>86</td>
<td>88.3</td>
<td>77</td>
<td>3.10</td>
<td>3.32</td>
</tr>
<tr>
<td>CNG$r_f$</td>
<td>152</td>
<td>480</td>
<td>117.9</td>
<td>4.11</td>
<td>4.11</td>
</tr>
</tbody>
</table>

The experimental backbone amide RDCs were fitted to EPAC2$f$, Rl$\beta_b$, Rl$\beta_f$, CNG$h_b$ and CNG$r_f$ CBDs obtained from PDB file 1O7F, 1RGS, 1RL3, 1VP6 and 1U12 respectively. The subscript f/b described the cAMP-free/bound state of the protein. $^a$ is the correlation value for linear fit of calculated versus experimental RDC; $^b$ represent the cAMP bound CBD; $^c$ Quality Q-factor; $^d$ number of experimental RDC used in the fit; $^h$ for the beta strand region residues showing high PFs were used (>6); $^e$ represent the apo state; * residues between 270 to 280 are used for analysis. h the residues in the $\beta6$ and $\beta8$ (PF <6) are used for fitting.

Table 2. Fits of cAMP free EPAC1b (149-305) RDCs.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\chi^2$</th>
<th>RMSD (Hz)</th>
<th>R</th>
<th>$Q^c$</th>
<th>N $d$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta^e$</td>
<td>PBC$^g$</td>
<td>Hinge$^h$</td>
<td>$\beta^e$</td>
<td>PBC$^g$</td>
</tr>
<tr>
<td>EPAC2$f$</td>
<td>36.6</td>
<td>120.63</td>
<td>73.35</td>
<td>2.01</td>
<td>4.4</td>
</tr>
<tr>
<td>Rl$\beta_b$</td>
<td>31.18</td>
<td>1.86</td>
<td>0.78</td>
<td>0.285</td>
<td>9</td>
</tr>
<tr>
<td>Rl$\beta_f$</td>
<td>83.59</td>
<td>3.04</td>
<td>0.414</td>
<td>0.467</td>
<td>9</td>
</tr>
<tr>
<td>CNG$h_b$</td>
<td>167</td>
<td>102.7</td>
<td>4.09</td>
<td>0.516</td>
<td>0.41</td>
</tr>
<tr>
<td>CNG$r_f$</td>
<td>152</td>
<td>67.9</td>
<td>3.9</td>
<td>0.422</td>
<td>0.667</td>
</tr>
</tbody>
</table>

$^{ah}$ is same as table 1. The blank cells did not have enough experimental RDC data required for reliable fitting using the program Pales.
Figure 4. Correlation between the experimental $^{15}$N-H RDC of EPAC1h(149-305) and the RDCs calculated from the crystal structure as mentioned in the Tables 1 and 2. Only selected
correlation plots are presented here as representative of the degree of fitting between the experimental RDC and calculated RDC. Panel a-i are the plots for the cAMP bound EPAC1 and panels j-o are those for apo-EPAC1. Panel a,d,g,j,m and o show the fitting of the observed RDCs for the β-strand region; b,e,h and k show the fitting for the PBC and c,f,i,l and n represent the fitting for the hinge region. The PBC and the Hinge of apo-EPAC1 (149-305) could not be fit to other structure due to absence of enough experimental RDC data.
Figure 5. Correlation between experimental $^{15}$N-H RDC of EPAC1h(149-318) and calculated RDC from crystal structure as mentioned in the Table 3 and 4. Only selected correlation plots.
are presented here as a demonstration of degree of fitting between the experimental RDC and calculated RDC. Panel a-i are the plots for the cAMP bound EPAC1 and panel j-q are the plots for apo-EPAC1. Panel a, d, g, j, m and p are the fitting of observed RDC for the β-strand region, b, e, h k, n and q shows the fitting for the PBC and c, f, i, l and o represent the fitting for the hinge region.

5.2.3 Solution structure of holo-EPAC1h (149-305)

The structure of holo-EPAC1h (149-305) was solved by a combination of 3D NOESY experiments, RDCs, H–bond constrains and dihedral angle constrains as explained in the material and method section. The structure statistics for the 10 best structures are presented in Table 5. Figure 6a shows the overlap of selected low energy structures of holo-EPAC1h (149-305) as explained in the method section (5.1.2.4). The holo-EPAC1 structure conserves the typical CBD fold with the eight stranded β-barrel forming a basket where PBC is positioned. However, in the solution structure the β2 does not have a very well defined secondary structure, which is also supported by the secondary structure predicted from the secondary chemical shift data (Fig. 3). A multitude of evidences including backbone HN compounded chemical shift data (Fig. 8) along with the recently published hydrogen exchange and secondary chemical shift data suggests that the cAMP binding affects the β2-β3 loop (134). Moreover, in the majority of the NMR structures calculated, the α3 helix becomes less defined and takes a loop like conformation. Consistent with these findings, recent comparative investigations of apo and holo EPAC1h (149-305) show that the cAMP binding affects the packing of the N-terminal helices and also destabilizes the α3 helix
In addition, the comparison of the apo EPAC2 and holo-EPAC1h structures shows that upon cAMP binding α3 moves closer to the β-barrel region (Fig. 6b).

| Table 5. Structure Statistics of 10 lowest energy structure of Epac1h (149-305) |
|---------------------------------|-------------------------------|
| NOE Distance Constraints        | 1762 (total NOEs)              |
| Intermolecular                   | 17                            |
| Intramolecular                   | 1745                          |
| Hydrogen bond constrains        | 59                            |
| Dihedral angle constrains       |                               |
| ψ                               | 49                            |
| Φ                               | 49                            |
| H-N RDC constrains\(^a\)        | 106                           |
| NMR constraint violations\(^b\) |                               |
| NOEs [Å]                        | 0.028 ± 0.005                 |
| Dihedral angles [°]             | 0.681 ± 0.133                 |
| R.m.s. deviation from idealized covalent geometry\(^b\) |                 |
| Bond lengths [Å]                | 0.012 ± 0.0004                |
| Bond angles [Å]                 | 1.53 ± 0.058                  |
| Improper dihedra angles [°]     | 1.89 ± 0.095                  |
| R.m.s. deviation for coordinate precision [Å]\(^c\) |     |
| Backbone atoms                  | 0.427 ± 0.133                 |
| All heavy atoms                  | 1.421 ± 0.31                  |
| Ramachandran Statistics\(^d\)  |                               |
| Residues in allowed region      | 95.2%                         |
| Residues in disallowed region   | 4.8%                          |

\(^a\) R

\(^b\) R.m.s deviation experimental data obtained from cns

\(^c\) Residues used form the r.m.s. deviations are 26-32, 40-52, 62-70, 71-76, 92-98, 101-104, 111-114, 117-120, 122-126, 134-137, 142-148, 149-155. The r.m.s deviation was calculated for the well defined secondary structure using the program Molmol(125).

\(^d\) Analyzed using FROCHECK-NMR (124).

The phosphate binding cassette of holo-EPAC1h shows the typical general architecture with a helix and a loop flanked by two β strands (β6 and β7), respectively. The PBC structural ensemble shows that the helix 5 spans both closed and open conformations as observed in the CNG structures (25) (Fig. 6c and Fig. 4 in Chapter 1). In addition binding of cAMP also increases the stability.
of the $\beta6$, $\alpha5$ and $\beta7$ in PBC in comparison to the apo structure (134). As predicted before by RDC analysis, upon binding to cAMP, the hinge helix moves closer to the PBC (Fig. 6d). This movement of the hinge region is necessary for the lid to move over the PBC as observed in PKA.

Figure 6. Solution structure of EPAC1$_a$(149-305). a) overlay of 10 lowest energy structure obtained after minimization in explicit solvent. The helixes are highlighted with red, beta-subdomains are with cyan and loops are with gray. The overlap of apo-EPAC2 CBD (cyan) and cAMP-bound EPAC1 (green) is shown in panel b. cAMP binding induces structural rearrangement in PBC, hinge and in the $\alpha3$ region. Panel d, highlights the movement of hinge region upon cAMP binding. The R1$\alphaB$ domain of PKA is represented as brown ribbon. The figure in panel ‘a’ was prepared using Molmol (125) whereas the rest of the figures are made using the program Pymol (13).
5.2.4 cAMP docking into the PBC of the Holo-EPAC1h (149-305) structure

It has been observed that cAMP is oriented differently within the CBDs of PKA, CNG and CAP (16, 25, 26, 57). The free cAMP exists predominantly in an anti conformation in equilibrium with a small population of syn conformation (16). In CAP and CNG, cAMP binds in an anti conformation, whereas in PKA cAMP is in the syn conformation (Figs. 3 and 6 Chapter 1). This conformational difference is critical for the CBD response to different chemical modifications of cAMP. For example, Rp-cAMPS, which is the only known PKA inhibitor, is actually an agonist for bacterial CAP protein. On the other hand the PKA agonist such as Sp-cAMPS is an antagonist for CAP. Therefore to comprehend cAMP signaling in EPAC, the orientation (i.e. syn or anti) of cAMP docking was determined from transfer NOEs measured with $^{13}$C$^{15}$N double filtered edited NOESY experiments (116).

The syn and anti conformations are defined by the orientation of the adenine base with respect to the ribose moiety. Table 6 summarizes the observed distance between the H1', H2 and H8 protons of cAMP with respect to the protons in the ribose moiety in the syn and anti conformations, respectively (Figs. 2a and Fig. 3 Chapter 1). The distance between H1' - H2' protons has been selected as a reference because the relative position of these protons does not change with the change in orientation of the adenine base. Since the syn and anti conformations depend on the relative orientations of the H2 and H8 adenine protons with respect to the ribose moiety, the NOEs involving these two protons probe the orientation.
of AMP. For instance, in the syn conformation the distance between H8-H1' is shorter than that in the anti conformation. Therefore, the syn conformation will generate a strong NOE signal between H8 and H1' along with a weak signal for H8-H2' and H2-H2' (Table 6, Fig. 7a). On the other hand, the anti conformation will give rise to medium to weak NOE signals between H8 and H3', H2', H1' and H5', respectively.

**Table 6. Average distances measured for the syn and anti conformation of cAMP**

<table>
<thead>
<tr>
<th></th>
<th>H1' (Å)</th>
<th>H2 (Å)</th>
<th>H8 (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Syna Silk</td>
<td>Anti</td>
<td>Syna Silk</td>
</tr>
<tr>
<td>H4'</td>
<td>2.81-3.02</td>
<td>2.8-2.91</td>
<td>6.15-6.68</td>
</tr>
<tr>
<td>H3'</td>
<td>3.69</td>
<td>3.68-3.73</td>
<td>3.91-3.99</td>
</tr>
<tr>
<td>H2</td>
<td>2.68</td>
<td>2.69-2.72</td>
<td>4.67-5.57</td>
</tr>
<tr>
<td>H1'</td>
<td></td>
<td></td>
<td>6.09-6.11</td>
</tr>
<tr>
<td>1H5'</td>
<td>4.61-4.63</td>
<td>4.56-4.62</td>
<td>3.95-5.24</td>
</tr>
<tr>
<td>2H5'</td>
<td>4.57-4.69</td>
<td>4.58-4.64</td>
<td>5.42-6.72</td>
</tr>
</tbody>
</table>

a distance for the syn conformation is obtained from the PDB entry 1RGS, 1NE4 and 1NE6  
b distance for the syn conformation is obtained from the PDB entry 1VP6, 1U12, and 1C6N  
c Hydrogen atoms were added using the program Molmol (125)

**Table 7. Relative NOE signal observed for free cAMP and cAMP bound to EPAC1**

<table>
<thead>
<tr>
<th></th>
<th>H1'</th>
<th>H2</th>
<th>H8</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>H4'</td>
<td>w</td>
<td>w</td>
<td>n</td>
</tr>
<tr>
<td>H3'</td>
<td>ov</td>
<td>ov</td>
<td>ov</td>
</tr>
<tr>
<td>H2</td>
<td>s</td>
<td>s</td>
<td>w</td>
</tr>
<tr>
<td>H1'</td>
<td>-</td>
<td>-</td>
<td>n</td>
</tr>
<tr>
<td>1H5'</td>
<td>w</td>
<td>w</td>
<td>s</td>
</tr>
</tbody>
</table>

A column represent the NOE for the cAMP bound to EPAC1h (149-305); B column represent the NOE for the cAMP bound to EPAC1h (149-318); C column represent the NOE for the free cAMP; s represent strong NOE; m represent medium NOE; w represent weak NOE; n represent no NOE observed; ov represent overlap peak
The cAMP resonances were assigned by 2D-homonuclear TOCSY experiments and the resonances of H2 and H8 were separated by comparing the spectra acquired for cAMP and d8-cAMP (Fig. 7c). The d8-cAMP was prepared by substituting hydrogen by deuterium at position 8 of the adenine base. This was done by heating the cAMP in D2O at 100 °C for 16 hours (136). Figure 7a and b and Table 7 show the relative NOE signals observed for the free cAMP and bound cAMP. For free cAMP strong NOE signal was observed for H8-H2', medium NOE signal for H8-H1', and very weak NOE signals were observed for H8-H5', H2- H2', H1'-H5' indicating an anti conformation. However, the strong H8-H1'NOE observed for bound cAMP suggests that cAMP binds to EPAC in a syn conformation (Table 7). The chemical exchange peaks between the free and the bound cAMP was determined by ROESY experiment as described in the method section. Our data also shows that the lid region as contained in the EPAC1h (149-318) construct has no effect on the orientation of bound cAMP (Table 7 and Fig. 7b).

The compounded chemical shift between apo and holo EPAC1h (149-305) (Fig. 8) reflects the formation of cAMP:EPAC contacts in the phosphate binding cassette (PBC, residues ~270-280). The structure shows that the phosphate group of cAMP is positioned at the N-terminus of α5 and the ribose moiety is sandwiched between the two ends of the PBC region. The equatorial oxygen of cAMP forms H-bonds with the conserved Ala272 and NHη of R279 (Fig. 7d). In addition, the interaction between the ribose ring and the protein is further
stabilized through a hydrogen bond between the axial oxygen of cAMP and Ala 280, and between 2'-OH and Gly269 NH. However our HD and HH exchange

Figure 7. cAMP docking at the PBC of EPAC1h. a) Cross section of $^{13}$C$^{15}$N filtered edited NOESY spectra of cAMP bound to EPAC1h (149-305). The transfer NOE peaks are labeled as a proton pair where as the NOE observed for the bound cAMP is suffixed with b. b) Cross section of the overlap ROESY spectra showing the chemical exchanged between the free (suffixed with f) and bound cAMP (suffixed with b). The spectra highlighted in black and red represents the EPAC1h (149-305) and EPAC1h (149-318) spectra, respectively. The magnitude of chemical shift for bound cAMP between the two construct is shown by dotted lines. c) The 1D spectra for the cAMP (top) and d8-cAMP (bottom).
Replacement of H8 with $^3$H8 has significantly reduced the H8 signal in d8-cAMP. d) represent the binding of cAMP to the PBC of EPAC1. The cAMP binds to EPAC in syn conformation where it forms multiple contacts with conserved G269, A272, R279 and A280 respectively. The surface represents total surface covered by each residue in the 10 representative low energy structures.

data suggest that these hydrogen bonds are only transiently stable when compared to PKA, accounting for the µM binding affinity of cAMP for EPAC (Fig. 9). On the other hand, the adenine base of cAMP is positioned in the vicinity of the β4-β5 regions, similarly to PKA (Fig. 7d). This is also evident from the cAMP dependent compounded chemical shift variations in the β4-β5 region due to aromatic ring current contributions arising from the adenine base of cAMP (Fig. 8).

5.2.5 Defining the intra-molecular signaling pathways originating from PBC

The structural comparison of apo & holo EPAC and the compounded chemical shift differences between the two states have shown that the cAMP signal propagates beyond the PBC and base binding regions (BBR) to well into the NTHB through the β2-β3 and the α6 regions (Figs. 6-8). This result was further validated by the cAMP dependent change in helicity of α3 (134) at the center of the NTHB and increased protection from solvent exchange observed upon cAMP binding for α2 and α4. Overall, we have observed an increased protection for the NTHB of EPAC upon cAMP binding indicating a tighter packing of the N-terminal helices in EPAC as compared to PKA (Fig. 9) (134). The high stability as implicated by the protection factors (PFs) observed for the residues in the α2 and α4 helices of the NTHB region is critical for the relay of
the cAMP signal to the ionic latch at the N-terminus of the CBD through a stable inter-domain coupling. In addition, an overall cAMP-induced PF enhancement has been observed for the β-strands residues of the CBD (Fig. 9). This group of highly buried sites with maximal PFs in the NTHB and the CBD relies on transient but cooperative global unfolding as opposed to local conformational fluctuations (134).

Figure 8. Compounded chemical shift difference between apo- and holo- EPAC1h (149-305) is plotted as a function of residue number in panel a. The secondary structure is indicated by black dotted
line where the positive values indicate α-helix and negative values indicate beta sheets. The red solid and dotted lines mark the average ± standard deviation. The residues showing significant changes in chemical shift (above average ± std) upon cAMP binding are also flagged by residue numbers. b) the chemical shift changes are plotted on the ribbon diagram of EPAC1h. The cyan and the green ribbon represent the apo EPAC2 and holo-EPAC1h structures respectively. The backbone amides showing significant chemical shift are shown by red ball and residues within the average ± std are represented by blue balls.

Figure 10 outlines the three sets of signals that originate from the PBC and propagate to the NTHB or the hinge region, as required to activate EPAC by removing the inhibitory effects of the ionic latch and of the switchboard region, respectively. The first set of ‘signaling contacts’ involves the hydrophobic interaction between the conserved L273 in α5 and F300 in α6 (65) as well as the hydrogen bond between K297 found at the N-terminus of α6 and A272 of PBC and the salt bridge between K297 and D276 (134). Upon binding to cAMP the hinge helix rotates to move towards the PBC. Due to this hinge movement the hydrogen bond between K297 and A272 is perturbed (Fig. 10a) and F300 moves approximately 1.3 Å towards the PBC. The side chain of F300 reorients to remove the steric hindrance between F300 and L273 (Fig. 10a). These observations were further independently validated by the compounded chemical shift change for the K297 and F300 upon cAMP binding (Fig. 8). A second set of interactions between the PBC and the NTHB is mediated through Q270 in the PBC and α3 in the NTHB. V204 (L204 in EPAC2) in α3 moves 4.2 Å towards the PBC upon cAMP binding, which brings α3 close to Q270 in the PBC (Fig. 8b). This observation is supported by the observed NOEs between the α3 and the β-subdomain (Table 8).
In PKA the residue corresponding to Q270 of EPAC1_h is E200 which forms a hydrogen bond with the 2' OH of cAMP. On the contrary, the side chain of Q270 in EPAC1_h faces away from the PBC and forms a hydrophobic contact with the α3 region. Previously detected secondary structure differences between apo and holo EPAC1_h as well as cAMP-dependent line broadening are consistent with this α3/Q270 interaction and point to a conformational ensemble in an intermediate exchange regime (134). This movement of α3 towards the PBC Q270 might contribute to dragging the rest of the NTHB towards the CBD and subsequently perturbing α1 thus releasing the EPAC inhibition through ionic latch.

**Figure 9. Plot of Hydrogen exchange protection factor (PF) for holo-EPAC1_h(149-305) as measured from H/D (a) and H/H (b) exchange experiments.** a) the secondary structures are indicated by red dotted lines. The residues for which H/D exchange rates could not be determined due to fast exchange are indicated by filled symbols close to the zero line. Up and down pointing arrows indicate that the reported PF value is a lower or upper limit, respectively. In both the panels the overlap peaks are shown by open symbols.
The third set of intra-molecular signaling pathways originating from the conserved R279 is mediated by the β2-β3 loop (134). Previous studies from our laboratory (Chapter 3) have shown cAMP-dependent changes in the compounded chemical shifts, 2° structure and solvent shielding observed for the β2-β3 loop (Fig. 3 & 8). Figure 10c, summarizes the allosteric network initiated by R279

Figure 10. Intra-molecular signaling pathway in EPAC. a) the first set of cAMP allosteric cross-talk involves A272, L273 and D276 in the PBC and the K297 in the hinge. The apo-EPAC2 structure is shown in cyan and holo-EPAC1h is represented by green ribbon. Upon binding to the cAMP the hinge rotates around the helix axis and moves closer to PBC by 1.3Å. Due to this movement the hydrogen bond (dashed line) between the K297 and the A272 & L276 breaks. b) second set of cross-talk involves direct contact between PBC and NTHB mediated through Q270 in PBC and V204 in α3. In the ribbon diagram apo-EPAC2, holo-EPAC1h and holo-R1αB are highlighted by cyan, green and brown color,
respectively. In PKA β200 forms H-bond with the 2'OH of cAMP, whereas in EPAC the residue at the similar position (Q270) interacts with the α3 helix which pulls the helix approximately 4.2Å towards the PBC. Two probable conformation of Q270 are represented by stick and the surface represents the van der Waals surface for all the conformation of Q270 in 10 low energy structures. c) the third set of contact is initiated from the conserved R279 in the PBC upon cAMP binding. R279 forms two hydrogen bonds (dashed line) with D236 and G235 in the β2-β3 loop through its backbone and interacts with S240 through its side chain. The β2-β3 loop in turn is connected to the α6 helix through backbone hydrogen bond between T239 and K297.

Table 8. NOEs between the α3 at the NTHB and the β-subdomain.

<table>
<thead>
<tr>
<th>α3</th>
<th>β-subdomain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A20:HN</td>
<td>G265Hα</td>
</tr>
<tr>
<td>A20:HN</td>
<td>I243Hα</td>
</tr>
<tr>
<td>A20:HN</td>
<td>I243 Hγ</td>
</tr>
<tr>
<td>K20:Hβ</td>
<td>G265 Hα</td>
</tr>
<tr>
<td>A20:Hβ</td>
<td>I243 Hγ</td>
</tr>
<tr>
<td>A20:Hβ</td>
<td>G265 Hα</td>
</tr>
</tbody>
</table>

* Data obtained from NOESY-HSQC Expt.

* Data obtained from 13C HSQC-NOESY-13C 13C HSQC Expt.

upon cAMP binding. The backbone amide of R279 forms a hydrogen bond to the carbonyl of D236 and in turn the amide of G235 forms a hydrogen bond to the carbonyl of R279. The hydrogen exchange experiment shows that cAMP binding actually stabilizes this interaction. Our previous work discussed in Chapter 3 showed that this network of H-bonds originating from the R279 is also very conserved in PKA. G235 on the other hand is further stabilized by a hydrogen
bond between the amide of D236 and the carbonyl of S233 in the β2-β3. In addition, the guanidinium side-chain of R279 forms a hydrogen bond with S240 located at the N-terminus of β3 (Fig. 10c). The β2-β3 loop is also connected to the α6 hinge helix through a hydrogen bond between the backbone of T239 and K297. Such cAMP-dependent perturbations of β2 and β3 also propagate to the adjacent β8 and possibly to the N-terminal helical bundle (NTHB) which is involved in the auto-inhibition of EPAC (134).

In conclusion, the solution structure of holo-EPAC1h (149-305) brought new light on the cAMP-dependent allosteric network of eukaryotic CBDs and explained how cAMP allosterically removes the steric inhibition of EPAC. This allosteric network involves both direct and indirect pathways that are analogous in PKA and EPAC, as both systems rely on the long range ‘cross-talk’ between the cAMP-binding site, the hinge helix, the β2-β3 region and the NTHB. Nevertheless, cAMP docking and the nature of the communication between the PBC and α6, in addition to the interaction between Q270 in the PBC and V204 in the NTHB, define unique characteristics for the cAMP allostery of the EPAC CBD.
Figure 11. Correlation of experimental residual dipolar coupling, measured for the holo-EPAC1<sub>h</sub> (149-305) (a, c, e, and g) and values predicted from the solution structure of holo-EPAC1<sub>h</sub> (149-305) obtained before (a) and after (c) water refinement, crystal structure of cAMP bound R1αB domain (PDB file: 1RGS) (e) and cAMP-free EPAC2 CBD (PDV file: 107F) (g), after best fitting the alignment tensors using the program Pales (119). The panel (b), (d), (e) and (f) represents the correlation between the experimental RDC measured for apo-EPAC1<sub>h</sub> (149-305) with respect to the predicted RDCs from the solution structure of holo-EPAC1<sub>h</sub> (149-305) obtained before and after water refinements, crystal structure of cAMP bound R1αB domain (PDB file: 1RGS) (e) and cAMP-free EPAC2 CBD-B (PDB file: 107F), respectively. The error on the fitting of the RDCs in panel (a), (b), (c) and (d) represent the standard deviation observed for the ten low energy structures.
FINAL COMMENTS AND FUTURE WORK

CHAPTER 6
6.1 **NMR spectroscopy represents a method of choice to investigate the highly dynamic CBDs**

cAMP-dependent allosteric changes have often been elusive to detailed characterization at atomic resolution due to the instability of either the holo or the apo state of CBDs, resulting in considerable experimental challenges in the direct structural comparisons between the two states. In the case of PKA and CAP, the apo vs. holo comparison was hampered by the instability of the apo state (*Chapter 2 and Chapter 3*). In *Chapter 2* we have proposed an NMR method for the comparative study of the holo and apo states of the regulatory subunit of PKA, which does not require a pure apo state. The residues undergoing exchange upon cAMP binding were mapped by CT-CPMG experiments (97, 98). In other words, the residues experiencing the on/off chemical exchange were 'spied' through the easily detectable cAMP-bound peaks by comparing two samples: one with excess cAMP and another with a slight cAMP deficient, causing a small population of the cAMP-free state. The proposed method is not only applicable to study cAMP:CBDs interactions, but it is also generally applicable to the rapid screening of small molecules against their respective binding partners.

Overall, our investigations have revealed that the key determinants of allostery in the CBDs are cAMP-dependent dynamic changes coupled with subtle yet functionally critical rearrangements of charge distributions and/or hydrophobic core packings. NMR
is exquisitely sensitive to these dynamical and structural variations and is therefore an ideal technique to investigate the cAMP-dependent allostery. Furthermore, it is now clear that the functions of CBDs are more adequately rationalized by an ensemble of multiple conformations in equilibrium with each other rather than by a single structure (64, 111, 138). Such intrinsic conformational heterogeneity typical of CBDs limits the resolution of their crystal structures and leads to high B-factors (9, 111, 139). For instance, no significant differences were detected by X-ray between the crystal structures of the R-subunit bound to the Sp-cAMPS agonist and to the Rp-cAMPS antagonist, despite the clearly different activities of these two cAMP-analogs (111). However, different allosteric network patterns between these two bound forms of the R-subunit were clearly revealed by NMR, leading to a model of cAMP-agonism and antagonism, as discussed in Chapter 4. It appears therefore that, while CBD crystal structures provide an invaluable initial framework for further biophysical investigations aimed at characterizing the nature of the cAMP-dependent allostery, NMR spectroscopy is a complementary technique useful for investigating the highly dynamic systems regulated by cAMP.

6.2 Understanding cAMP allostery in PKA and future challenges

To understand the structural and dynamical basis of allostery, agonism and antagonism in PKA, we have focused on the cAMP binding domain A of R1α, due to its pivotal role in controlling the activity of the catalytic subunit. Activation of PKA by CBD-A has been explained on the basis of a three-shell allosteric model (Chapter 3),
which in turn led to a *model of agonism and antagonism* (*Chapter 4*). In the three-shell allosteric model, the 1\textsuperscript{st} shell of interactions involves primarily the PBC and the direct interactions that stem from it, including a cAMP-dependent salt-bridge between the PBC and the C-interacting C-helix (Fig. 4a, *Chapter 3*). However, the 1\textsuperscript{st} shell alone does not account for the full cAMP dependence of PKA activation and a 2\textsuperscript{nd} shell must be considered (Fig. 4b, *Chapter 3*). The 2\textsuperscript{nd} shell contains V162 and R226, which interact with two key relay sites in the β2-β3 loop (I163 and D170). Recent systematic structural genomic analysis has shown that the β2-β3 loop has co-evolved with the residues in the PBC (140). Furthermore, the residues in the hydrophobic core of the β-subdomain comprising I175, M180, V213, V162, F198, and Y173 are conserved in all cyclic nucleotide binding (CNB) domains or CNB-like domains. Both I163 and D170 are in contact with a highly conserved PBC arginine (R209) that anchors the cAMP phosphate, but they have different allosteric functions. The D170 acts as a cAMP-dependent electrostatic relay switch that controls the tertiary structure of the α-subdomain (141, *Chapter 3*), whereas the I163 site translates the cAMP release into a perturbation of the CBD-A hydrophobic core (3\textsuperscript{rd} shell) promoting partial unfolding and uncoupling of the α/β-subdomains. Consistent with this model, preliminary biochemical characterizations of the I163A mutant of R1α in our laboratory showed reduced thermal stability for the mutant in comparison to holo- CBD-A (Fig. 1). However, a complete biochemical and biophysical study of this mutant will be essential to decipher the effect of the I163A mutation on the cAMP allostery. The intra-molecular signaling pathways relayed by I163 and D170 are both fully activated by cAMP agonists, while cAMP antagonists do not
activate these allosteric networks. Such differential activation patterns allowed us to provide the first rationalization of agonism and antagonism in a CBD (Chapter 4). We have also shown that the dynamics of PKA CBD-A ensures that the conformational switches controlled by cAMP are fully reversible.

![Graph](image)

**Figure 1. Thermal denaturation profiles of RIα (119-244).** Thermal denaturation for wild type RIα:cAMP (black), apo (red), Rp-cAMPS bound (green), D170A (blue) and I163A (violet) mutants were monitored over 30°C to 80 °C by CD spectroscopy (AVIV model 215 instrument). 300μg/ml protein in NMR buffer was scanned at 222nm using a 1-mm quartz cell. The molar ellipticity [θ] at each temperature was computed by [q]=OD x (weight in mg/ml/Mol.Wt. x No. of a.a. residues)⁻¹, where OD is the measured optical density.

Our previous NMR investigations of CBD-A (RIα 119-244) alone do not provide a full picture for the activation of PKA by cAMP, which involves PKA CBD-B as well. A complete allosteric model of the R-subunit should also account for the cross-talk between CBD-A and -B. A recent crystal structure of the R:C complex points towards a salt bridge between R366 and E261 as a vital link for the cooperativity between the two domains and
to CBD-B:C contacts (63). However, several central questions are still open on the communication between these two CBDs. What are the interaction networks that link the cAMP binding sites of CBD-A and -B in PKA? What is the dynamical basis for such CBD-A/B cross-talk? In addition, it is known that the Rp-cAMPS antagonist affects the communication between domains A and B (111) and therefore the additional question arises as to what the role of the CBD-A/B inter-dependence is in the cAMP-agonism and antagonism. One possible path mediating the communication between sites A and B might involves a series of charge relays that starts with R333 in PBC:B and ends with E200 in PBC:A and the hydrophobic core of CBD-B, mediated by the methylenes of R333. The latter interaction evolving from the $\alpha$ and $\beta$ subdomains has been found recently to be highly conserved among all CNB or CNB-like domains (140).

6.3 Role of water molecules in cAMP signalling: Hydration study of PBC in PKA and EPAC

The role of water molecules in biomolecular structure and dynamics has long been recognized. Water has been described as the “lubricant of life” that plays a pivotal role in protein folding, ligand binding and enzyme catalysis (142-144). Goldbeck and coworkers have shown the global kinetic implication of water molecules in haemoglobin allostery, through their study of conformational fluctuations as function of viscosity and osmotic stress (145). However the dynamic of water molecules in allostery at atomic resolution is still not fully understood. High resolution NMR is an excellent technique to study the
functional hydration of proteins at atomic resolution. The NMR pulse sequences used to investigate hydration are generally comprised of three blocks (144). In the first block, the water magnetization is selected and in the second block the magnetization is transferred to probe protein protons through dipole–dipole cross relaxation and/or chemical exchange. In the third block the signal is detected through homo or hetero nuclear frequency labeling. The NMR based hydration experiments have been extensively reviewed by Haung and Melacini (2006), Wider (1998) and Otting (1997) (144, 146, 147). Currently we are investigating the allosteric role of water molecules in the regulatory subunits of PKA and EPAC1 as model systems. The sequence alignment of the PBC in different cAMP binding domains shows that this region is conserved among different cAMP binding domains ranging from bacteria to mammal (Fig. 2). The structural alignment among the cAMP bound PBC crystal structures from PKA, CAP, HCN and CNG confirms not only the structural conservation, but also reveals the conservation of water molecules connecting the cAMP allosteric hot spots (as described in Chapters 3, 4 and 5) (Fig. 2). For instance, the three water molecules present in the region between R209, D170 and G169 of R1a (119-244) are very conserved in all PBCs (Fig. 2a) (Chapter 3). Interestingly all these residues belong to the primary shell of allosteric signaling. Therefore, it might be possible that the dynamics of these water molecules is critical for the cAMP signal propagation. Table –1, summarizes the physical properties of selected water molecules in the PBC of R1a (119-244) bound to either the Sp-cAMPS agonist or cAMP. Similarly, Table – 2, and Table – 3 summarize the physical properties of the same water molecules in the Rp-cAMPS-bound (antagonist) and R:C
complex crystal structures, respectively (63, 111). The increase in SASA values calculated using single water molecules listed in Table 3, suggests that the water is more dynamic in the cAMP free state and exchanges rapidly with the bulk water, whereas in the ligand bound form the dynamics of these water molecules are more restricted.

Figure 2. Structure alignments of PBC and conserved water molecules. The structure superimposition of PBC (carton) of a) R1α-A (blue) and R1α-B (orange) domain, b) R1β-A domain, c) R1β-B domain, d) CAP, e) HCN f) CNG in cAMP bound form are represented here. The water molecules are indicated by sphere, where blue represent the R1α-A and orange represent the other PBCs. The residue corresponds to R209, N171, 1163 and Q164 are represented as stick. The structures were aligned with the program pymol (13), by selecting the PBC region only for the R1α- A & B (PDB ID 1NE6), R1β – A & B (PDB ID 1CX4),
Table 1. Buried or Cleft Water Molecules in Rtc (119-244):Sp-cAMPS and Comparison with Experimental Hydration NMR Data

<table>
<thead>
<tr>
<th>Water</th>
<th>All H2O</th>
<th>One H2O</th>
<th>Number of Hydrogen Bonds</th>
<th>B-F- / Å⁺</th>
<th>Probe H2O⁺</th>
<th>Observed Normalized WNOE/sp-cAMPS Bound, cAMPS-Bound</th>
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<tr>
<td>W701 A</td>
<td>0.1</td>
<td>3.1</td>
<td>1 (P208 O)</td>
<td>2 (T207 OG1, Sp S)</td>
<td>(47)</td>
<td>A210 (2.9)</td>
</tr>
<tr>
<td>W701 B</td>
<td>3.5</td>
<td>5.8</td>
<td>1 (V162 O)</td>
<td>2 (Dl70 OD1, Y173 O)</td>
<td>(42)</td>
<td>N171 (3.8)</td>
</tr>
<tr>
<td>W701 C</td>
<td>2.5</td>
<td>6.9</td>
<td>1 (E168 O)</td>
<td>2 (Dl70 OD1, R209 Ne)</td>
<td>(43)</td>
<td>R209 Ne (1.9)</td>
</tr>
<tr>
<td>W702 D</td>
<td>6.4</td>
<td>6.4</td>
<td>3 (G169 N, G206 &amp; T207 O)</td>
<td>0</td>
<td>(45)</td>
<td>G169 (2.1)</td>
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<tr>
<td>W700 E</td>
<td>3.8</td>
<td>9.9</td>
<td>1 (S197 O)</td>
<td>2 (S197 OG1, E200 OE1)</td>
<td>(41)</td>
<td>---</td>
</tr>
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</table>

Solvent-accessible surface areas (SASA) were computed using the program Naccess (113) with a probe radius of 1.4 Å. Residues 119-244 of the Ine6 structure solved at 2.3 Å resolution (111) were considered in the analysis. For each buried or cleft water molecule the SASA was calculated both in the presence ("All H2O") and in the absence ("One H2O") of the other water molecules. This SASA is < 10 Å² for buried or cleft water molecules. Number of potential hydrogen bonds based on protein nitrogen or oxygen atoms (reported in parenthesis) within a 3.2 Å radius from the water oxygen atom in the Ine6 structure. Buried or cleft water molecules are characterized by either at least two backbone hydrogen-bonds or one backbone hydrogen-bond and at least two side-chain hydrogen-bonds. Surface hydrogen-bonds, including interactions with other independently identified buried or cleft water molecules. Side-chain hydrogen-bonds, including interactions with the CAMP ligand or its analog. Water molecule B-factors in the Ine6 structure, reported in parenthesis because the average B-factor for the buried protein atoms lies outside the normal range of 10 - 20 Å². Protein probe H2O⁺ atoms are considered within a 4.0 Å radius from the water oxygen atom. Ratio of cross-peak intensities in the water-selective NOE and the HSQC spectra normalized to the value observed for R209 Ne in each state. Exchange peaks identified in the CLEANEX-PM experiment (112) are denoted by Ex⁺.

Table 2. Buried or Cleft Water Molecules in Rtc (119-244):Rp-cAMPS and Comparison with Experimental Hydration NMR Data

<table>
<thead>
<tr>
<th>Water</th>
<th>All H2O</th>
<th>One H2O</th>
<th>Number of Hydrogen Bonds</th>
<th>B-F- / Å⁺</th>
<th>Probe H2O⁺</th>
<th>Observed Normalized WNOE/sp-cAMPS Bound, cAMPS-Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>W701 A</td>
<td>1.5</td>
<td>1.5</td>
<td>1 (P208 O)</td>
<td>2 (T207 OG1, Rp O)</td>
<td>(26)</td>
<td>A210 (2.8)</td>
</tr>
<tr>
<td>W702 B</td>
<td>4.0</td>
<td>7.3</td>
<td>1 (V162 O)</td>
<td>3 (Dl70 OD1,2, Y173 O)</td>
<td>(42)</td>
<td>N171 (3.9)</td>
</tr>
<tr>
<td>W706 C</td>
<td>4.2</td>
<td>9.0</td>
<td>1 (E168 O)</td>
<td>1 (R209 Ne)</td>
<td>(48)</td>
<td>R209 Ne (2.0)</td>
</tr>
<tr>
<td>W701 D</td>
<td>5.7</td>
<td>5.7</td>
<td>3 (G169 N, G206 &amp; T207 O)</td>
<td>0</td>
<td>(45)</td>
<td>G169 (2.0)</td>
</tr>
<tr>
<td>W702 E</td>
<td>4.3</td>
<td>11.0</td>
<td>1 (S197 O)</td>
<td>2 (S197 OG1, E200 OE1)</td>
<td>(29)</td>
<td>---</td>
</tr>
</tbody>
</table>

As for Table 1 but using the Ine4 structure solved at 2.4 Å resolution (111). Broadened beyond detection. Despite the presence of only one side-chain potential hydrogen bond this water molecule is still considered here for comparative purposes. Despite this SASA > 10 Å² this water molecule is still considered here for comparative purposes.
Table - 3. Hydration Analysis for RIA (119-244) Bound to the C-Subunit of PKA

| Water  | All H₂O | On: H₂O | Number of Hydrogen Bonds^c | B-F, Å² | a: SASA / Å² \n|:-----|:------|:-----|:---------------------|:------|:---------------|
| W91A  | 1.3   | 30.7 | 1 (A210 N) | 0     | (45)          |
| W108B | 9.0   | 21.9 | 0         | 1 (Y173 O) | (56)         |
| W52C  | 2.5   | 16.6 | 1 (E168 O) | 1 (R209 NE) | (47)         |
| W330D | 1.5   | 10.5 | 3 (G169 N, T207 N & O) | 0     | (49)          |
| W115E | 9.1   | 23.2 | 1 (S197 O) | 2 (S197 OG, E200 O2E) | (51)      |

^a As for Table 1 but using the R-subunit chain in the 1u7e structure solved at 2.0 Å resolution (55).

The preliminary NMR hydration data of the regulatory subunit of PKA bound to cAMP, Sp-cAMPS and Rp-cAMPS indicate that the water molecules W701, W756, W762 and W713 are more dynamic than W718, which forms a hydrogen bond with the NHε of R209 (Table I & II). However, the direct comparative study of hydration has been severely hampered by the instability of the cAMP free regulatory subunit of PKA. The hydration of CBDs in both apo and holo forms could be understood from the EPAC 1h (149-318) construct, because this protein is stable in both forms. However, the bottleneck at this point is the lack of a high-resolution crystal structure for holo-EPAC 1h (149-318). Hence it is essential to solve first the crystal structure of holo-EPAC 1h (149-318).

6.4 Evolution of cAMP allostery from PKA to EPAC

Our comparative examination of PKA and EPAC CBDs reveals that the function of these domains depends on the long range propagation of the cAMP signal from the PBC to the active sites. The CBD loci that are connected by the cAMP-dependent allostERIC network are the PBC, BBR, NTHB, β2-β3 loop, and the hinge regions. A
systematic genome analysis of 7000 different CBDs illustrates the importance of a conserved cAMP allostery pathway extending from the PBC to the NTHB and mediated through the β2-β3 loop (140). Residues G269, A272, R279, and A280 in the PBC of EPAC1 are evolutionarily conserved. The conservation of the cAMP allostery signal originating from R279 through β2-β3 is further supported by a similar response for both CBDs to the Rp-cAMPs antagonist and the Sp-cAMPs agonist (110). Nevertheless, thorough examination of the sequences of PKA and EPAC CBDs indicates subtle yet functionally significant differences between these two domains.

Both Figure 2 in Chapter 1 and Figure 1 in Chapter 5 show that the highly conserved E200PKA is substituted by Q or K in EPAC1 or EPAC2, respectively. Mutation of Q270E in EPAC1 does not improve the cAMP binding, however the reciprocal E200Q mutation in PKA significantly decreases the cAMP affinity (10). In PKA E200 forms a hydrogen bond with the 2’OH of the ribose of cAMP (Fig 10b, Chapter 5). In contrast, the corresponding residue in EPAC1, Q270, faces away from the PBC in the holo state and interacts with the α3 helix in NTHB (Chapter 5). The promiscuous role of this amino acid residue in position 270 of EPAC explains the different response towards the 2’substitution of cAMP (110). For example, in the case of EPAC, the 8-pCPT-2’OMe- cAMP selectively functions as a super activator. In addition, our H/D and H/H exchange data (Chapters 3 and 4) shows that, unlike PKA, the hydrogen bonds formed by cAMP with G269 and A280 in EPAC are only transiently stable. This further explains the μM dissociation constant of cAMP for EPAC in comparison to the nM dissociation constant observed for PKA. However, in the holoenzyme complex, PKA has a similar dissociation
constant for cAMP to that of EPAC (69), since in the R:C complex, the PBC of PKA takes an open conformation as observed in EPAC or the apo-CNG structure (26).

![Diagram](image)

**Figure 3.** cGMP conformation in EPAC1\textsubscript{b} (149-305) was determined by \textsuperscript{13}C\textsuperscript{15}N filtered edited NOESY spectroscopy. The weak H\textsubscript{8}-H\textsubscript{1'} NOE peak with respect to H\textsubscript{8}-H\textsubscript{3'} indicates that cGMP is in anti conformation (see Table 5 in Chapter 5).

The CBDs of PKA and EPAC also differ in their responses to modifications at the N6 position in the adenine base of cAMP (110). For instance, N6-substituted cAMP analogs like cGMP, cIMP, cXMP, and 6-Phe-cAMP do not affect the PKA activity, while in EPAC they function as antagonists. This implies that the N6 in the adenine base plays a significant role in cAMP allostery of EPAC. Our NOE based experiments show that
cGMP binds to EPAC in an *anti* conformation whereas in most of the CBDs, such as PKA and HCN, it binds in a *syn* conformation (26, 139) (Fig. 3). In addition, our NMR data reveals that in PKA the cGMP-induced chemical shift changes are similar to those observed for cAMP (Fig. 4a). In PKA, cGMP only affects the A210 and A211 differently from cAMP, because the O6 of the guanidine base is positioned in the vicinity of the PBC C-terminus and of the β7 N-terminus (139). Unlike PKA, the binding of cGMP to the

---

**Figure 4. Effect of N6 substitution on cAMP allostery.** a) Representation of the compounded chemical shift between cAMP with respect cGMP-bound Rlα (119-244). b) Plot of compounded chemical shift difference observed between cAMP and cGMP in the EPAC1α (149-318) construct. The black dashed line represents the average chemical shift. The secondary structure of the Rlα (119-244) is shown as a dotted line, where positive and negative values represents α-helix and β-sheet respectively.
EPAC CBD negatively affects the allosteric hot spots in the BBR, the hinge and the NTHB region (Fig. 4b). Again it has been reported that mutations in the BBR of CAP, for which similarly to EPAC cGMP is also an antagonist, alter the ability of CAP to discriminate between cAMP and cGMP (17). Therefore it might be possible that in CAP and EPAC the BBR plays a significant role in cyclic nucleotide recognition or in sensing the allosteric potential of the bases. Another difference between the cAMP allostery networks of PKA and EPAC is the direct cross-talk between Q270 and the α3 helix in EPAC, which is absent in PKA. A similar cross-talk between the PBC and the N-terminal helices has been proposed to be critical for the cAMP-dependent gating of HCN (24). However, in the PKA regulatory subunit such contact between the α3 and PBC is facilitated only in the presence of the catalytic subunit (55).

Concluding Remarks. In summary, the cAMP allosteric mechanism proposed in this thesis not only explains how cAMP signaling propagates from the PBC to other functional regions in PKA and EPAC, but also explains the mechanism of cAMP antagonism. Currently, allosteric proteins such as kinases, 7TM receptors and phosphodiesterase are being investigated as potential therapeutic targets (149). For example, the recently developed ‘cinecalcet’ functions as a positive allosteric modulator of the calcium sensor belonging to class-C 7TM receptors (149). One advantage of utilizing allosteric regulatory domains as drug targets is their specificity (150). Generally the allosteric modulators within a family of enzymes are not as conserved as their functional sites, thus opening new opportunities to design specific allosteric modulators (150). Along these lines, it is possible that the subtle differences between the CBDs of EPAC
and PKA emerged in this thesis as well as the proposed allosteric role of bound water molecules will facilitate the development of cAMP analogs as potential drug leads.
Model-free Analysis of Backbone Dynamics of RIA (119-244).

The backbone dynamics of the cAMP bound regulatory subunit of PKA – domain A (RIA 119-244; see Chapter 4) was further analyzed using the model-free approach. This method utilizes the relaxation data from two fields and provides quantitative values for the generalized order parameter ($S^2$), R_{ex}, and $\tau_c$. However, the model fitting depends on the accurate determination of the overall correlation time ($\tau_c$), which is a function of the shape of the molecule as represented by the rotational diffusion tensor ($D_{\|}/D_\perp$) (151). The theoretical background of this approach has been discussed previously in Chapter 1.

The $R_1$ and $R_2$ relaxation rates and NOE were measured at two different fields: 700 MHz and 600 MHz. The experimental conditions were the same as described in Chapter 4. The 10% trimmed average values of $R_1$, $R_2$, and NOE are shown in Table 1.

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<tr>
<th>Field, $T$</th>
<th>$n^a$</th>
<th>$^{15}$N($^1$H)NOE</th>
<th>$R_1$, s$^{-1}$</th>
<th>$R_2$, s$^{-1}$</th>
<th>$\tau_c$ (ns)$^b$</th>
<th>$D_{|}/D_\perp$</th>
<th>$^{ns}x$</th>
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<tbody>
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<td>14</td>
<td>57</td>
<td>0.825</td>
<td>1.699</td>
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<td>7.0 ± 0.019</td>
<td>1.347 ± 0.028</td>
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<td>0.805</td>
<td>1.483</td>
<td>11.351</td>
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</table>

$^a$ Number of residues with NOE >= 0.65. The residues with NOE less than 0.65 were removed before averaging. $^b$ The overall correlation time and Dpar/Dper ratio were calculated using the program ‘Quadric’ and further optimized by using Model-free 4.15r (79).
The model-free calculations and model selections were done as described by Mandel et al. (1995) using the ModelFree 4.15 program provided by Prof. Arthur G. Palmer (79). The initial estimations of the rotational diffusion tensor and overall correlation time ($\tau_c$) were obtained from the $R_2/R_1$ ratios using the program quadric_diffusion (152). The structural coordinates required for the regulatory subunit (R1α 119-244) were obtained from the Protein Data Bank file 1RGS and the H- atoms were added using Molmol (57, 125). For the initial run, the $\tau_c$ and the diffusion tensor were determined from the $R_2/R_1$ ratios for the residues with NOE greater than 0.7. The calculation was further refined by successive elimination of $\tau_c$ for the residues with $\chi^2 > 25$ in the first selection, $\chi^2 > 16$ in the second selection and $\chi^2 > 9$ in the third selection, respectively. The diffusion tensor was further optimized using the model 1 by excluding residues with NOE less than 0.65 and fixing the N–H bond length and $^{15}$N CSA to 1.02Å and -170 ppm, respectively. Model selection was performed to fit the relaxation data either to one parameter (S2 model 1), two parameters ($S^2$, $\tau_c$ or $S^2$, Rex models 2 and 3 respectively) or three parameters ($S^2$, $\tau_c$, Rex or $S^2$s, $S^2$t, $\tau_c$ models 4 and 5) using an axial diffusion model with $\tau_c$ fixed at 7 ns and $D_///D_\perp$ at 1.347. Uncertainties in the fitting of the dynamic parameters were determined by a 500 step Monte Carlo simulation. It was observed that three residues, Y120, A129 and Y244, did not fit to any model, therefore no motional parameter could be determined for them.

Table-2 summarizes the optimum model selection and the optimized model-free parameters for the backbone $^{15}$NH. Most of the backbone amide protons have $S^2$ values
greater than 0.85 with the exception for the residues at the C-terminus and at one of the allosteric relay sites described in Chapter 3 (i.e. D170). The residue D170 that functions as an electrostatic switch has an $S^2$ value of $0.749 \pm 0.103$. The $S^2$, $\tau_c$ and $R_{ex}$ values are plotted in Figure 1 as a function of residue number. The residues at the Xn-helix, D170, N171, PBC and the B/C helix show significant $R_{ex}$ contributions, which correlate well with our proposed model of a transiently stable ternary complex based on the residual dynamics in the cAMP-bound state (Chapter 4). However, the residual dynamics of the regulatory subunit also contribute to the ambiguity in the determination of $D_\parallel/D_\perp$ ratio. Since the model-free calculation depends on the accurate determination of the correlation time, the error in the determination of the $D_\parallel/D_\perp$ ratio will influence also the fitting of the relaxation rates to any of the Model-free models. We have therefore analyzed the relaxation rates based on the reduced spectral density approach (85, 88). This approach is independent of any model and the relaxation rates are used to estimate the density functions ($J(\omega)$) at selected frequencies, as described in Chapter 1.

Table 2. Summary of backbone dynamic parameters of cAMP:RIα(119-244) from Model-free calculations

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Res. No.</th>
<th>Model</th>
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<th>Error</th>
<th>$\tau_c$ ns</th>
<th>Error</th>
<th>$R_{ex}$</th>
<th>$S^2$</th>
<th>Error</th>
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Figure 1. Model-free analysis for cAMP bound R1α (119-244). a) Generalised order parameters (S²) b) the internal correlation time (τc) and c) the chemical exchange contribution (Rex) are plotted against the residue number. The secondary structure of the R1α (119-244) is represented by the dotted lines.
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