

cAMP Allostery in Epac

cAMP ALLOSTERY IN EXCHANGE PROTEIN DIRECTLY ACTIVATED BY cAMP

By

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TITLE: cAMP Allostery in Exchange Protein Directly Activated by cAMP

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Abstract:

Cyclic-3',5'-adenosine monophosphate (cAMP) is an ancient signaling molecule that is found in a variety of species from prokaryotes to eukaryotes and translates extra-cellular stimuli into tightly controlled intra-cellular responses. The two major mammalian cAMP sensors are protein kinase A (PKA), for the phosphorylation of the downstream effectors, and the exchange protein directly activated by cAMP (Epac), for the guanine nucleotide exchange in the small GTPase Rap proteins. In this study, we investigated the intra-molecular cAMP dependent allosteric network of Epac cyclic nucleotide binding domain (CBD) via solution NMR spectroscopy. Epac proteins have been shown to employ an auto-inhibition strategy in the control of the equilibrium between the active and the inactive states. In the absence of cAMP, the periphery of the Rap recognition site is masked via an ionic interface provided by the N-terminus of the CBD. Binding of cAMP at the distal Phosphate Binding Cassette (PBC), results in weakening of this interface. Here we show that the cAMP binding signal is propagated to the sites important in Epac activation, *i.e.* the ionic interface, via two key allosteric spots within the CBD. We have also determined the dynamics as a key carrier of cAMP effects to the region forming the ionic interface (ionic latch). Hence entropic enhancements emerged as a key effector in the cAMP mediated ionic latch weakening. We have also provided initial evidence of a negative allosteric contribution from the C-terminal Hinge-Lid region (CHLR) on the cAMP induced Epac activation. In addition to these findings, we also observed critical differences in the mode of cAMP recognition and inter-subdomain communication between the Epac and PKA. A detailed understanding of these two ubiquitous systems, will aid in the development of agonists and antagonists that are relevant in the drug lead development for related diseases, such as Alzheimer's and diabetes.

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List of Abbreviations:

BBR,	Base binding region
cAMP,	Adenosine 3',5'-cyclic monophosphate
CBD,	cAMP-binding domain
CDC25-HD,	CDC25 Homology domain
CHLR,	C-terminal Hinge-Lid Region
CNG,	Cyclic nucleotide gated ion channel of LotiK1
CR,	Catalytic region
CRP,	cAMP receptor protein
DEP,	Disheveled-egl-10-pleckstrin
Epac,	Exchange protein directly activated by cAMP
GEF,	Guanine-nucleotide exchange factor
H-D,	Hydrogen - Deuterium exchange
HCN	Hyperpolarization-activated, cyclic-nucleotide-modulated channel
hEpac,	Human Epac
HSQC,	heteronuclear single-quantum coherence
NMR,	Nuclear magnetic resonance
NMRD,	NMR dispersion
NOE,	Nuclear overhauser effect
NTHB,	N-terminal helical bundle
PBC,	Phosphate binding cassette
PKA,	cAMP-dependent protein kinase A
PF,	Protection factor
RA,	Ras-association domain
REM,	Ras exchange motif
RI α ,	Isoform I α of the regulatory subunit of PKA
RR,	Regulatory region
STD	Saturation transfer Difference

1.1 Introduction:

It has been fifty one years since Rall and co-workers have discovered and purified a heat stable factor in the liver homogenate that stimulated the liver phosphorylase formation (1). Two years later this factor was characterized as cyclic-3',5'-adenosine monophosphate or cAMP (2). cAMP acts as a second messenger molecule in response to extra-cellular stimuli such as epinephrine and glucagon. Binding of these hormones to the extra-cellular portion of the 7-transmembrane G-coupled receptors induce a conformational change that results in exchange of GDP to GTP of the peripherally coupled heterotrimeric stimulatory G-protein and hence its activation. Activation of the G-protein is followed by conformational changes that dissociate the G_α subunit from $G_{\beta\gamma}$. The resulting G_α subunit will interact and activate the adenylate cyclase, which produces cAMP from ATP (Figure 1A). The signal is terminated when GTPase activating proteins stimulate the hydrolysis of GTP to GDP and the re-formation of the heterotrimeric G-protein. Elevation of cAMP results in a multitude of cellular responses such as phosphorylation, ion channel regulation and guanine nucleotide exchange activities. Hence this ancient second messenger can modulate a variety of physiological effects from memory development to hearth rhythm, from insulin secretion to cellular junction formation and from cell adhesion to cell growth (3).

1.2 Effectors of cAMP:

Due to its conserved role as a second messenger molecule in a variety of species, several proteins capable of sensing this cyclic nucleotide and producing a biological response have been selected during the course of evolution. Bacteria such as *Escherichia Coli* (*E. coli*) and primitive eukaryotes such as *Saccharomyces cerevisiae* have as sole receptor for cAMP, the cAMP receptor protein (CRP) and serine/threonine protein kinase

A (PKA), respectively (4, 5). In higher eukaryotes PKA still remains the major sensor of cAMP but other critical receptor proteins have also been discovered such as cAMP regulated ion channels (6) and more recently the guanine nucleotide exchange factor for the small GTPases Rap 1 and 2, called Epac, *i.e.* 'Exchange protein directly activated by cAMP' (7, 8).

CRP is a 45 kDa dimer, with each subunit composed of two domains, a larger N-terminal cAMP binding domain (CBD) and a smaller C-terminal DNA binding domain (Figure 2A). The two subunits form an extensive dimer interface via helix C of the CBD in each subunit (9, 10). cAMP contacts primarily the α C, β 4 and β 5. Mutational investigations have suggested that ligand recognition and binding results in structural perturbations in α B and α C that not only change the orientation of the two subunits but also the orientation of each domain within the subunits with respect to each other (11). CRP is known to regulate catabolite-sensitive operons in *E. coli* encompassing over 150 genes and its function can be modulated via interaction with cAMP (12, 13). cAMP drastically increases the affinity of this proteins toward DNA. Bound to cAMP, the DNA binding domain will be in a new conformation that allows for specific DNA recognition of sequences near the RNA polymerase binding sites (14). CRP binds major grooves on DNA via a helix-turn-helix motif of the DNA binding domain, which introduce a strain in the DNA molecule by $\sim 90^\circ$ bending (14). It has been proposed that this results in the opening of the DNA molecule, which facilitates RNA polymerase binding. *E. coli* lives under continuously changing environments, hence selection and regulation of carbohydrate sources is key to its survival. This organism "prefers" glucose as its carbon source,

however upon reduction of glucose in the medium, the cAMP levels are enhanced within *E. coli* that will activate *lac* operon for utilization of lactose via CRP (15).

Cation conducting channels such as hyperpolarization-sensitive cyclic nucleotide-modulated channels (HCN) and cyclic nucleotide-gated channels (CNG) can be regulated by cAMP. These channels couple the intra-cellular second messenger concentration with the control of membrane potential, hence allowing the cell to regulate the electrical gradient across the membrane for proper physiological function (16). Both channels contain similar tetrameric structures with the four subunits arranged in a symmetrical circle (15, 17). Each subunit for both HCN and CNG channels contains an N-terminal hydrophobic membrane spanning domain, a linker region and a C-terminal CBD (Figure 2C). HCN channels can sense changes in membrane potential via an Arg rich helical segment embedded in the membrane spanning region of the protein (17). This paddle-like structure is termed 'voltage sensor' and can initiate the gating current, which is the result of perturbations of charged amino acids upon conformational rearrangements of the gate of the ion channel (15, 17). Binding of cAMP has been shown to enhance the sensitivity of HCN toward changes in membrane potential by decreasing the threshold potential required for channel opening (15). Also it has been proposed that cAMP binding to CBD might increase the diameter of the cytosolic face of the gate by rearranging the linker region (17). In this regard CBD plays an inhibitory role in the absence of cAMP and it has been shown that deletion of CBD produce levels of activity similar to channels with CBD in the presence of cAMP (15). In the case of CNG, the channel is insensitive to any membrane potential and can only be activated via cAMP, which will exert a pulling/pushing force on

the last helix of the membrane integrated domain via conformational rearrangements in the N-terminal helical bundle (NTHB) of its CBD (18).

PKA is a Ser/Thr kinase and is ubiquitously expressed in mammals and, with the exception of plant phyla, is a conserved signaling pathway in all eukaryotes (19). PKA is a heterotetramer composed of two catalytic and a dimer of regulatory subunits. The regulatory subunit contains a leucine zipper like dimerization domain, a pseudo-substrate sequence and two CBDs, CBD-A and CBD-B from the N- to the C-terminus, respectively. In the absence of cAMP, the regulatory subunit forms high affinity interactions via an extended interface that positions the pseudo-substrate sequence in the catalytic cleft of the C-subunit, which is unable to accept γ -phosphate due to the presence of an Ala residue instead of Ser/Thr (20). This conformation keeps the kinase inactive by sterically excluding the catalytic cleft from its substrates. Recently Susan S. Taylor and co-workers have elucidated the atomic resolution structures of the regulatory subunit of PKA bound to cAMP and to the C-subunit separately (19-21). In the C-subunit bound form the R-subunit adopt an extended dumbbell shape conformation, which contact the C-subunit with an approximate area of $3800 \text{ \AA}^2$, mostly contributed via CBD-A. CBD-A contacts the C-subunit via a hydrophobic interface provided by its NTHB, phosphate binding cassette (PBC) and the C-terminal B/C helix (Hinge). Comparison of the C-subunit and cAMP bound structures of R-subunit have allowed for a molecular explanation for the stepwise pathway for PKA activation (19). First, cAMP binds the CBD-B since the PBC of CBD-A is buried in the R:C interface and not accessible for ligand binding. In addition, W260, which is involved in stacking interaction with the base of cAMP, is trapped $30 \text{ \AA}$ away from the PBC via a salt bridge formed between E261 and R366 between the C-terminal

helices of CBD-A and -B, respectively (Figure 2B). Second, upon ligand binding to CBD-B, Y371 stacks against the base of the nucleotide and this results in the disruption of the aforementioned salt bridge. Third, the stacking interaction results in the movement of the C-terminal helix toward the PBC and weakens the interaction between CBD-B and the C-subunit. The disruption of the salt bridge also frees the W260 from the R:C interface and destabilizes the extended C-terminal helix of the CBD-A. This results in W260 approaching the PBC of the CBD-A to interact with the second incoming nucleotide. Fourth, the stacking interaction between the cAMP base and W260 stabilizes the ligand binding to CBD-A, however it is still unknown if binding of the second cAMP requires dissociation of the pseudo-substrate sequence from the C-subunit since, in order for the cAMP to bind to the PBC of CBD-A, it must be dislodged from the C-subunits. Step five; PKA is activated with the complete dissociation of the R and C subunits.

Epac is a single polypeptide chain, multi-domain protein that causes nucleotide exchange in Rap 1 and 2 from the GDP-bound inactive to the GTP-bound active forms (22). Up to this date there are two known isoforms of this protein, Epac1 and Epac2. Through sequence homology, the domains of this protein were predicted and later confirmed by X-ray crystallography of mouse Epac2 (23, 24) to be organized in a regulatory region (RR) containing a DEP (Dishevelled, Egl-10, Pleckstrin) domain for membrane localization, a CBD-B for cAMP recognition/binding and a catalytic region (CR) containing a RA (Ras Association) domain that is proposed to bind to the members of Ras family, a REM (Ras Exchange Motif) domain and a CDC25-HD (CDC25 Homology Domain) from the N- to the C-terminus respectively. REM interacts with and stabilizes the CDC25-HD, which is responsible for nucleotide exchange in Rap proteins (25). Epac2

contains an extra CBD domain N-terminal to the DEP domain with presently undefined function and is termed CBD-A (Figure 2d). Approximately eight years after the discovery of Epac proteins, the first atomic resolution structure of full length Epac2 from *Mus musculus* was solved in the absence of cAMP (24). The structure revealed the mechanism of Epac auto-inhibition in which the N-terminal RR sterically blocks the predicted periphery of the Rap binding site in CDC25-HD. The structural details of the interaction between Epac and Rap proteins, resulting in the exchange of GDP for GTP, are still elusive but the crystal structure of a homologous complex namely that of Ras bound to its exchange protein Son of Sevenless (SOS) has been determined (26). SOS catalyzes the nucleotide release by inserting into Ras a α -helix resulting in the displacement of the phosphate binding P-loop in the switch 1 region (26). This reorganization leads to the opening of the orifice of the GDP/GTP binding site. At the same time, the side chains presented by the inserted helix create a chemically unfavorable environment for the phosphate group of the nucleotide, further catalyzing the release of GDP (26). Since SOS does not interfere with the binding site of the ribose and base of the nucleotide, this results in the Ras-SOS complex to adopt a conformation in which the nucleotide can be released and rebound. The superposition of the catalytic domain of SOS in complex with Ras on to the catalytic domain of Epac shows only minor deviations in 3D structure, suggesting that the Epac mediated activation of Rap1 and 2 might proceeds via a similar mechanism (24). Further support came from mutation of a conserved Arg in the CDC25-HD, which was predicted to be critical in contact with Rap, to Ala that resulted in significant loss of exchange activity of Epac protein (24).

1.3 Biological significance of Epac:

Since its discovery in 1998, which was stimulated by the observation of insensitivity of cAMP-induced Rap activation to inhibitors of PKA, Epac has been increasingly shown to be a critical player in a variety of tissues from brain to heart, from kidney to liver and from sperm cells to endothelial cells (15, 25, 27).

Both Epac and PKA act independently to down regulate the activity of sodium-potassium ATPase exchanger 3 (NHE3) in the brush border membrane of proximal tubules of the kidney (28) which play a critical role in the regulation of not only cellular but also systemic pH. The role of Epac in kidney is not confined to NHE3 regulation. Analysis of perfused inner medullary collecting duct (IMCD) via confocal fluorescence microscopy has shown that an Epac-specific agonist (8-pCPT-2'-O-Me-cAMP), in the presence of PKA inhibitors, induced calcium mobilization and exocytosis in IMCD, which results in increased insertion of aquaporin-2 in IMCD to regulate osmotic water permeability in the kidney collecting duct (29).

Epac1 has been shown to be involved in the regulation of cell adhesion and cell-cell junction formation mediated by Rap1. β 1-integrin mediated cell adhesion to fibronectin was observed in Ovar3 cells via stimulation with 8-pCPT-2'-O-Me-cAMP. Upon down-regulation of Rap1, the observed adhesion effect was inhibited (30). In endothelial cells, elevation of intra-cellular cAMP concentrations via extra-cellular stimuli such as prostoglandins, has been shown to reduce cell permeability. Both PKA and Epac are expected to relay the cAMP signal to cell-cell junction and in the case of Epac, 8-pCPT-2'-O-Me-cAMP stimulated junction maturation resulting in decreased cell permeability (31-34). Further evidence for the involvement of Epac in junction formation was the inhibition

of junction maturation upon down-regulation of Epac expression with small interfering RNA (siRNA) for Epac1 (34) and inhibition of Rap via Rap GTPase (32).

Epac proteins have also been implicated in the process of glucose-mediated insulin secretion. The imported glucose, via GLUT2 transporter, increases the intra-cellular ATP concentration that will down-regulate the ATP dependent potassium channels (27, 35, 36). The resulting change in the membrane potential activates the voltage-dependent calcium channels that not only increase the intra-cellular calcium concentration but also enhance the release of calcium from intra-cellular calcium stores (35, 36). The increase in calcium, stimulates insulin containing vesicles to fuse with plasma membrane for secretion. Three lines of evidence suggest the involvement of Epac2 in insulin secretion process. Mutation of both cAMP binding sites of Epac2, that reduced cAMP affinity, and down-regulation of Epac2 expression with siRNA, reduced insulin secretion (37). It has also been shown that 8-pCPT-2'-O-Me-cAMP can increase glucose-mediated insulin secretion (38). It has been postulated that Epac2 exerts its effect on insulin secretion via three different paths. First, it has been shown that Epac2 interacts with Rim2, which assists docking of intra-cellular vesicles to the cytosolic face of the plasma membrane (37). Hence Epac2 might prime intra-cellular insulin containing vesicles for secretion. Second, 8-pCPT-2'-O-Me-cAMP stimulated release of calcium, was inhibited upon de-sensitizing Epac2 for cAMP via mutation (38), suggesting that Epac2 might control calcium channels. Third, 8-pCPT-2'-O-Me-cAMP inhibited ATP dependent potassium channels, while N6-Benzoyl-cAMP (*i.e.* Epac antagonist and PKA agonist) did not have any inhibitory effect (39-41).

Epac proteins, along with PKA, are also known to be critical players in hearth tissue. Two different effects of Epac have been observed in cardiomyocytes. First, there are

data suggesting the Epac1 controls ERK5 kinase mediated cardiac hypertrophy. Scott and co-workers (42) have identified a multi-protein complex in the neonatal cardiomyocytes formed by muscle Specific A Kinase Anchoring Protein (mAKAP). mAKAP recruits PKA, PDE4D3 (phosphodiesterase), and Epac1 at the perinuclear membrane (42). PKA phosphorylation of PDE3D3 at Ser 13 and 54 enhances the recruitment of the phosphodiesterase to the mAKAP complex and metabolism of cAMP, respectively (42). Epac1 association with the complex is mediated by PDE4D3. High local cAMP concentrations can activate Epac1 which in turn will down-regulate ERK5 phosphorylation of PDE4D3 that reduces the cAMP metabolism. Hence both Epac1 and PKA are coordinated by the mAKAP complex to reduce local cAMP concentrations. The Epac mediated down regulation of ERK5 kinase is repressed at low cAMP concentrations, allowing for cardiac hypertrophy (42, 43).

Both Epac and PKA have been also implicated to play critical roles in Alzheimer disease (AD). The expression levels of Epac1 and 2 is altered in the regions of brain that are associated with the disease such as frontal cortex and hippocampus, and are unchanged in regions of brain that are resistant to the disease pathology such as cerebellum (44). In addition, activation of Epac enhanced the processing and release of the amyloid precursor proteins (APP) ectodomain, which exerts memory enhancements and neuroprotective effects (45, 46). PKA activation on the other hand has a negative impact on the AD. The microtubule associated protein, tau, is involved in microtubule assembly and is abundantly found in the neuron tissue (47). Hyperphosphorylation of tau via PKA can result in formation of helical and paired filaments in neuron cells (47), which is a hallmark of AD

pathogenesis. AD is a classical example that highlights the necessity for cAMP analogs that can distinguish between Epac and PKA.

Finally Epac has also been shown to be critical in processes that are affected in cancer cells such as cell migration and cell shape. Epac mediated activation of Rap1 is enhanced upon interaction between Epac and light chain 2 (LC2) of the microtubule associated proteins (MAP) (48). It has been suggested that this observation can be the result of LC2 mediated stabilization of microtubules since Nocodazole (inhibitor of microtubule polymerization) mediated inhibition of Rap1 activity is reverted by transfection of cells with LC2 (49). Also direct LC2 mediated conformational changes on the CBD of Epac1 may also be a factor in the enhancement of Rap1 activity since LC2 enhanced the ability of Epac1 to interact with cAMP-agarose (49).

1.4 Universal features of CBDs:

All CBDs share a common fold containing two subdomains, a continuous β -subdomain and a non-continuous α -subdomain. The former consists of eight continuous beta strands with a small separation in the continuity introduced by the PBC helix between $\beta 6$ and $\beta 7$ and the subdomain adopts a beta sandwich topology (Figure 1B). The latter consists of the NTHB, the PBC, the Hinge and the Lid. The α -subdomains of different CBDs are more diverse compared to their β -counterparts both in terms of sequence and structure. For instance the NTHB can be composed of four helices (*e.g.* Epac) to only of one helix (*e.g.* CRP) and the orientation of the helices can be different from one protein to the next (50). NTHB in almost all cases forms at least a portion of the interface between the regulatory and the catalytic regions of the protein. Another hyper-diverse segment of α -subdomain is the Lid, which is proposed to stack against the base of the cAMP and

protects it from solvent and phosphodiesterases (51). The region of the domain that is proposed to be the Lid is very poorly conserved in sequence and can be either a helix, such as in PKA, or a beta sheet, such as in Epac in the absence of the ligand.

A recent genomic and structure based search has identified several conserved features shared by all known CBDs (50, 52). First, sequence alignment of different CBD containing proteins from variety of species has shown that in addition to the PBC there is also an additional element within the β -subdomain that is conserved within the CBD superfamily. The $\beta 2$ - $\beta 3$ loop, which is in proximity to the PBC, has been shown to contain a conserved Gly residue which can contact the conserved Arg at the C-terminus of the PBC (52). It has been proposed that these two residues have co-evolved and help to connect the cAMP binding sites to distal allosteric spots (52). A structural search for conserved spatial patterns has also found the NTHB, the $\beta 2$ - $\beta 3$ loop, the PBC and the Hinge to be conserved three dimensional features of all CBDs with the exception of CRP which contains a single helix in NTHB (50). It has been proposed that CRP may compensate for the lack of this element via formation of a homodimer in which the Hinge of one domain is the binding partner of the PBC of the opposite domain (50).

1.5 Proposed mechanism of Epac activation:

According to the crystal structure (24), in Epac proteins there are two contact points between the RR and the CR that define the relative orientation of the RR and the CR (Figure 2D). The first contact is at the C-terminus of CBD-B, which is a rigid tripartite β -sheet-like structure, called switch board, with tight backbone to backbone contacts (24). The middle strand of this motif contains a conserved VLVLE sequence that upon mutation to AAAAAA, results in a protein that is constitutively active even in the absence of cAMP

(53). This shows that the integrity of the switch board is essential to the precise coordination of the RR for the steric inhibition of CR and Rap interactions. The second contact point is the ionic interactions between the NTHB of CBD-B, containing four helices, and the CDC25-HD in the periphery of the Rap binding site. N-terminal truncation that disrupted these salt bridges resulted in an active Epac in the absence of cAMP (24). The two aforementioned mutations also hinted at the possibility that Epac exists in a dynamic equilibrium between the active and inactive states regardless of the presence or absence of cAMP. These speculations were later supported by the observation of a double conformation behavior in the NMR resonances of the isolated CBD-B of Epac1 in the absence of cAMP (54). cAMP binding stabilizes the active conformation, thus shifting the equilibrium toward the active form.

To date there is no atomic resolution structure of the cAMP bound Epac due to poor solubility and cracking of the crystals upon soaking in to cAMP solution (23); hence the precise activation mechanism of this protein remains largely elusive. Nevertheless, through mutational insights and knowledge of the structural response of other known CBDs to cAMP via crystallographic studies, a mechanism of Epac activation has been proposed (15, 24, 27).

The CBD-B of Epac adopts the universal fold of CBDs, namely the eight stranded β -barrel (contiguous β -subdomain) containing the PBC where the cAMP docks. This subdomain is nested within a non-contiguous α -subdomain which contains the NTHB, PBC and the C-terminal Hinge helix. The NTHB forms the RR:CR interface by harboring key ionic latch residues Q168, D172 and E197 in $\alpha 1$ and $\alpha 2$. These residues are conserved in both Epac iso-forms and are known to mask the periphery of the Rap binding site (24).

Comparison of the structures of the cAMP bound and free CBDs of the PKA regulatory region iso-form R1 α (PKA-R1 α) and LotiK1 channel, have consistently demonstrated that the C-terminal Hinge helix swings toward the PBC in the core of the β -subdomain upon ligand docking to the PBC (18-21). The consequence of such a structural response is two fold; first, this inward movement will result in perturbation of the NTHB and second, the stacking interaction of the Lid, a helix C-terminal to the Hinge, against the base of the cAMP, provides protection against the solvent and phosphodiesterases. In Epac the NTHB helices α 3 and α 4 face the Hinge region and are expected to be affected by the inward motion of the Hinge (Figure 1B). Indeed, significant cAMP-dependent chemical shift variations, which are sensitive probes for structural perturbations, were observed for residues facing the Hinge region (55, 56).

In the cAMP free Epac, the PBC orientation orients a conserved Leu (L273) thus sterically blocking the inward movement of the Hinge as L273 counteracts Phe (F300) in the Hinge helix. Re-orientation of the PBC helix upon ligand binding will disrupt this hydrophobic contact, paving the way for the Hinge to swing/rotate toward the PBC. Mutation of the L273 to a bulky Trp residue has shown to stabilize the inactive conformation, possibly by hindering this inward motion (23). It has been proposed that this movement results in a rigid body rotation of the RR away from the CR, allowing the access of Rap to CDC25-HD for nucleotide exchange (24). Due to the extended backbone to backbone contacts of the switch board it is possible that the overall structure of this motif remains intact upon ligand binding and it will play as a pivot for the rigid body rotation (24). This mode of response to cAMP will allow the first strand of the switch board to come within close proximity to the base of the nucleotide for stacking. Indeed mutation of

a His residue (H317) in the switch board, which is solvent exposed in the absence of cAMP, to Ala resulted in 5-fold reduction in maximal activity (22, 53). However this stacking interaction still remains to be proven directly since the H317A mutation did not affect the affinity of Epac toward the cAMP (22, 53) and recent molecular simulations have suggested that the H317 orientation upon ligand binding does not allow proper stacking with the base of the cAMP (4).

1.6 The purpose of this thesis:

The goal of this thesis is to prepare and optimize CBD of human Epac1 for high resolution nuclear magnetic resonance (NMR) investigation in order to elucidate the intra-molecular communication network of this domain in regards to cAMP. This work sought to examine the dynamics and structural perturbations introduced by docking of the ligand to the PBC, which help us to understand not only how cAMP binding is reported to the R:C interface but how this reported message results in weakening of the interface. We also sought to compare this model of binding and allostery to that of the CBD-A in the evolutionary related PKA isoform RI α (PKA-RI α). A detailed picture of ligand recognition and allostery is vital to the development of analogs that can distinguish between Epac and PKA for both *in vivo* research and therapeutic efforts. In undertaking this endeavor, we have used variety of NMR techniques such as chemical shift mapping, hydrogen-deuterium (HD) exchange and ^{15}N -relaxation measurements. In addition we have employed urea denaturation and point and stepwise truncation mutations, in the investigation of the cAMP allostery in Epac CBD.

1.7 References:

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1.8 Figure Captions:

Figure 1) Intra-cellular cAMP signaling. A) The overall cAMP action is depicted in a simple diagram. First messenger such as epinephrine can be sensed by extra-cellular face of the G-protein coupled receptor (GPCR). This results in the conformational change in the cytoplasm portion of the receptor, which will induce the exchange of GDP to GTP in the heterotrimeric G protein. Activation of this peripheral cytoplasmic protein via GTP binding induces the intra-cellular domain of adenylyl cyclase to convert ATP to cAMP. Increase in the cellular cAMP concentration can be recognized by cAMP binding domain (CBD) containing proteins such as PKA, Epac, CNG and HCN, which translate the signal to multitudes of biological responses. The signal is attenuated via hydrolysis of GTP to GDP with the subsequent re-formation of the inactive heterotrimeric G protein. Phosphodiesterases can also decrease the cAMP concentration. In PKA, Epac and CNG/HCN, the CBD containing subunits of the proteins are colored red and the catalytic (PKA, Epac) or the transmembrane (CNG, HCN) subunits are colored blue. B) The CBD of Epac2 is represented with Hinge, PBC, $\beta 2$ - $\beta 3$ loop, base binding region (BBR) and NTHB containing the ionic latch (56).

Figure 2) Structure gallery of major CBD containing protein types in prokaryotes and eukaryotes. All the structures are made with Pymol software using PDB files: 1CGP (CRP), 2QCS and 1RGS (PKA left to right), 1Q43 (HCN) and 2BYV (Epac). A) The structure of a complex between CRP-cAMP and a double helix DNA is shown with the kink introduced by the DNA binding domain of the protein. The CBDs are shown in green and the DNA binding domain in cyan. The double stranded DNA is shown in van der Waals surface in brown. The cAMP is shown with stick representation. B) The structures of C-subunit and cAMP bound PKA is shown with CBD-A, CBD-B and C-subunits in green, cyan and brown (van der Waals) respectively. The extended and kinked conformation of C-terminal helix of CBD-A in C-subunit and cAMP bound states are highlighted with dash lines respectively. W260, responsible to stack against cAMP base in CBD-A is masked by C-subunit. The E261-R366 salt bridge in C-subunit bound state is highlighted in red circle. C) The asymmetric unit structure of the N-terminal truncated channel bound to cAMP is shown with CBD in green and connecting helices (to the membrane pore) in brown. Perturbations in the NTHB of the CBD via cAMP binding are proposed to affect the conductance of the channel. D) The three dimensional structure of EPAC2^(PDB: 2BYV) is shown with CBD-A, DEP, CBD-B, REM, RA and CDC25-HD colored in yellow, green, red, orange, brown, cyan. The red circle marks the switch board region and the blue circle the ionic latch. The dark blue in CDC25-HD is proposed to cause nucleotide exchange in Rap proteins. The dash line represents the regulatory (top) and catalytic (bottom) halves of Epac.

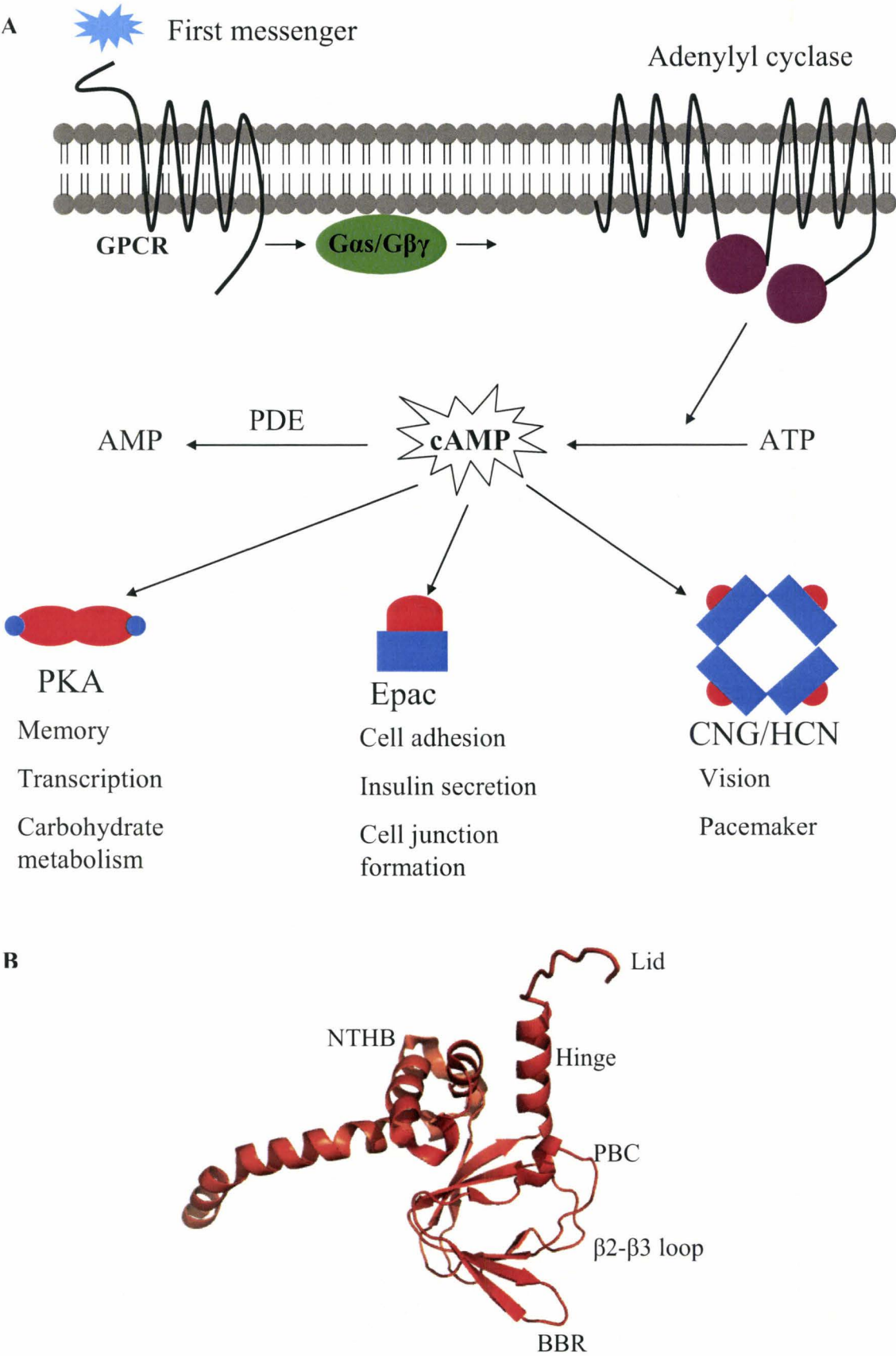
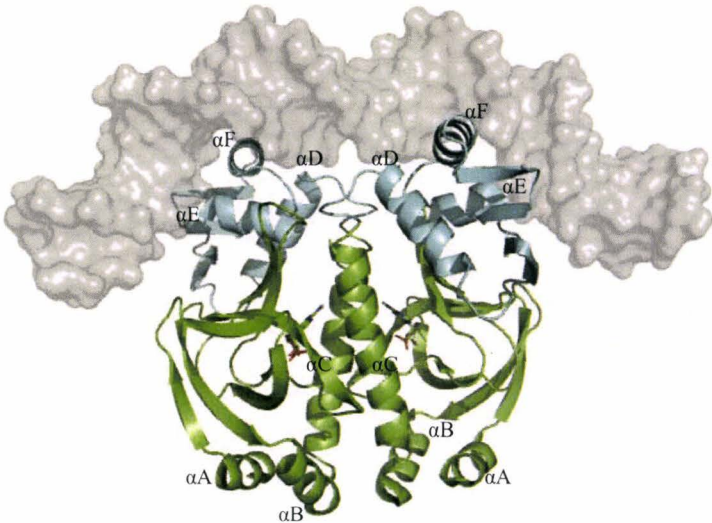
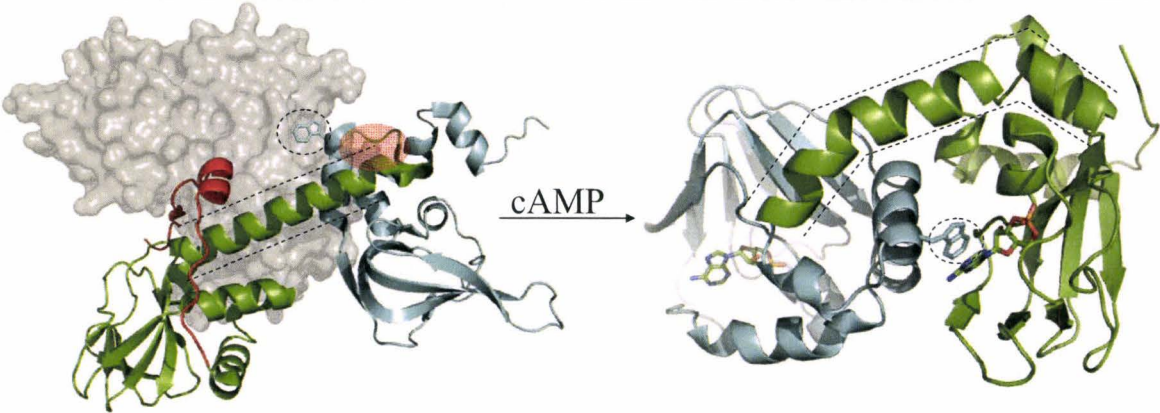


Figure 1

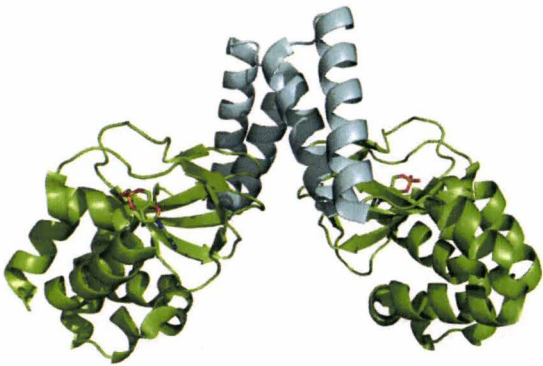
A



B



C



D

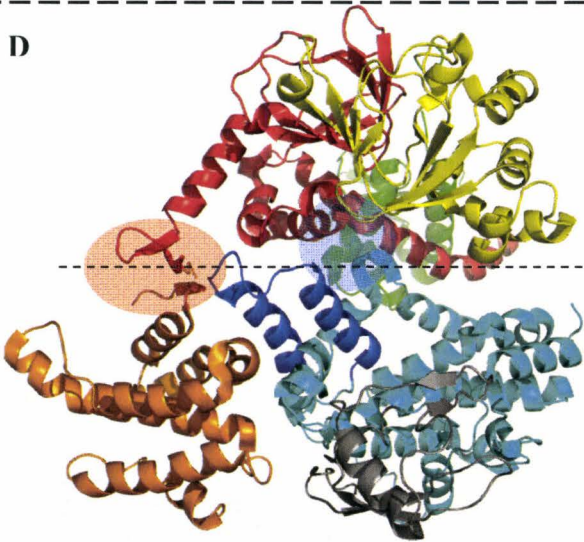


Figure 2

2.1 Intra-Molecular cAMP Allosteric Pathway:

In this chapter we prepared a construct of Epac1 cyclic nucleotide binding domain (CBD) that is known to form an ionic auto-inhibitory interface with the periphery of Rap binding site on Epac and sterically inhibits the association of Rap with Epac catalytic domain for nucleotide exchange. The protein was soluble in both cAMP free and bound states providing an excellent opportunity for the investigation of the cAMP allostery in human Epac1 that was not possible for the homologous CBD of PKA-R1 α isoform (1-3). In the case of PKA the unstable ligand free state was indirectly investigated by formation of a minor population of ligand free state that constantly interchange to the liganded state to ensure its stability (1). Hence, equipped with both stable cAMP bound and free states in the case of the Epac, an unprecedented picture of allostery can be obtain. According to the crystal structure of the ligand free Epac2 (Chapter 1, Figure 2d) there can not be a direct communication between the ligand binding site (PBC) and the ionic interface between the RR and CR due to distant constrains. Hence the information of cAMP binding must be reported to this interface via an intra-molecular network of interactions. This chapter sought to examine this signaling pathway by comparative analysis of the human Epac1 (149-318) that comprises of an N-terminal helical bundle (NTHB), harboring the ionic latch residues to contact the CR, an 8-stranded β -barrel that contain a helix-loop motif forming the PBC, the Hinge helix and the C-terminal Lid region (Chapter 1, Figure 1b). Our investigation showed that the previously proposed hydrophobic contact between the PBC and the Hinge via L273-F300 bridge is part of much larger interaction network that not only involves the aforementioned contact but also contains polar/ionic contact between the PBC and Hinge helix. We also showed that the radiation of cAMP signal from PBC is not limited only to Hinge helix but also involved the β 2- β 3 loop within the β -subdomain.

These two cAMP relay sites are then in contact with the NTHB and can inform this motif regarding the cAMP binding event. This study also revealed significant differences in cAMP recognition and allostery between PKA and Epac systems, which might be useful in the future generation of novel cAMP based analogs that can activate one sensor and be insensitive to the other, hence providing a therapeutic potential for diseases that are related to one of these cAMP sensors.

2.2 Experimental procedures:

2.2.1 Protein Preparation: Human Epac1 149-881 DNA fused with pGEX-4T3 expression vector was used as a template in polymerase chain reaction (PCR) to insert a stop codon at position 319 with appropriate primers. *E. coli* strain XL1-blue was transformed with the new DNA and extracted (QIAGEN protocol). A sample of DNA was sequenced to ensure the stop codon insertion. The resulted DNA was then transformed in to *E. coli* BL21 (DE3) expression strain for protein expression. The cells were grown at 37 °C until the OD_{600nm} of 0.55-0.6 were reached, at which the protein expression was induced with 1 mM IPTG at 37 °C for 3.5 h. The cells were harvested using centrifugation at 8000 ×g for 10 min at 4 °C and stored at -80 °C. When needed, cells were thawed on ice and re-suspended in lysis buffer (PBS, 10 mM EDTA, 10 % v/v glycerol, supplemented with protease inhibitors). The cells were lysed using French Press and centrifuged for 40 min at 20,000 ×g for 1 h at 4 °C. The supernatant was incubated with pre-equilibrated Glutathione Sepharose 4 Fast Flow beads for 4 h at 4 °C with slow rotation. The beads were then washed with 0.5 M NaCl and 50 mM Tris (pH 8) five times. The Epac protein was cleaved from the GST tag using biotinylated thrombin (NOVAGEN) for 16 h at 4 °C in 50 mM Tris (pH 7.6 with MES), 50 mM NaCl and 1 mM DTT followed by thrombin removal via

Streptavidin beads with slow rotation. Further purification was achieved with the ion exchange chromatography using a Q-column (GE Health Sciences). Finally the protein was dialyzed against the NMR buffer (50 mM Tris at pH 7.6 with Mes, 50 mM NaCl, 1 mM TCEP, 0.02 % w/v NaN_3 , 5 % v/v D_2O) and concentrated to 0.1 to 0.5 mM, unless otherwise specified. To obtain cAMP-bound Epac1 (149-318), 2 mM cAMP (Biolog Life Sci. Inst.) was added to the final purified protein. The purified sample was examined with 1D proton NMR and ^{15}N heteronuclear single-quantum coherence (HSQC) spectrum to assess its feasibility for high resolution NMR and its response to cAMP (Figure 1c&d).

2.2.2 NMR Measurements: All NMR spectra were collected at 34 °C with a 700 MHz spectrometer equipped with a TCI cryo-probe. The temperature calibration was performed with both a thermocouple and an ethylene glycol sample. The HSQC spectra were collected with 128 (^{15}N) and 1024 (^1H) complex points and spectral widths of 14.2 and 31.8 ppm for the ^1H and ^{15}N dimensions, respectively. ^1H chemical shift calibration was done using DSS proton signal followed by indirect calibration of the ^{15}N ppm using the nitrogen to proton gyro-magnetic ratio (4). The carrier frequencies of the proton and nitrogen channels were centered on the water resonance and on the middle of the backbone amide region of the protein, respectively. GARP4 pulse train was used to decouple ^{15}N with a 1.32 kHz radio frequency pulse (RF) strength. All 2D spectra were processed for analysis using NMRPipe and/or Xwinnmr (Bruker, Inc.) (5). Cross-peak fit heights were measured with Sparky 3.111 (6) and Gaussian line fitting, unless otherwise indicated. The backbone resonance assignments were performed with the standard triple resonance experiments (*i.e.* HNCO, HNCA, HN(Co)CA, CBCA(Co)NH, HBHA(Co)NH, HNCACB, and HNHAHB) (3). All 3D spectra were processed with NMRPipe (5) and analyzed by

Sparky 3.111 software (6). The secondary structural probabilities were determined using the secondary chemical shifts with the PECAN software (7).

2.2.3 Hydrogen-Deuterium Exchange: H/D exchange experiments were carried out on a 0.25 mM uniformly ^{15}N Epac (149-318) prepared by 10 times dilution with a 99.9% D_2O NMR buffer of a 2.5 mM sample in the H_2O NMR buffer (50 mM HEPES, 50 mM NaCl, 1 mM TCEP, and 0.02% w/v NaN_3). The dead-time of the experiment, which is the time elapsed between the first exposure to D_2O and the start of the first HSQC spectrum, was about 20 min. The H/D exchange rates were measured by a total of 120 HSQCs, with the first 30 spectra accumulated only two scans and the rest, four scans. The total time of spectral acquisition was ~18 h. In addition, the peak intensity of the slowly exchanging amides was monitored with four scan HSQCs for several days after initial D_2O exposure. The resulting resonance intensities were examined with NMRPipe using a 3×3 matrix centered at each peak maximum. Errors on the resonance intensities were estimated with the standard deviation of the spectral noise. The rates of exchange were calculated via the Levenberg-Marquardt nonlinear least-square exponential fitting using the Curvefit software (8). Covariance matrix and Monte Carlo Simulation was used to calculate the errors for the exchange rates (9). Alanine polypeptides were used to calculate the intrinsic exchange rates using the SPHERE program (10). The protection factor values were calculated as the ratios of the intrinsic to the observed exchange rates and are reported here in logarithmic scale.

2.2.4 Sequence and Structure Analysis: Sequence alignment was done with CLUSTALW sequence aligning software (11), while Pymol (12) was used for the structural analyses.

2.2.5 Urea unfolding: Urea unfoldings were performed with 5 μ M protein in 5 mM Hepes and 50 mM NaCl for both free and 150 μ M excess cAMP. Proteins with different Urea concentrations (0, 1, 2, 3, 3.5, 4, 4.4, 4.8, 5.2, 5.6, 6, 6.4, 6.8, 7.2, and 7.6 M Urea) were excited at 293 nm and the emission spectra were recorded from 300-450 nm using TECAN flourmeter. The fractional denaturations were calculated as previously described (13, 14). In brief; the maximum intensity of the emission spectrum for 0 and 7.6 M Urea were used to calculate the fractional unfolding with the following equation (14):

$$Fu = 1 - [(R_M - Ru) / (Rf - Ru)] \quad (1)$$

Where R_M is the ratio of the intensity at various urea concentrations, and R_f and R_u represent the values of this ratio at 0 and 7.6 M urea respectively. A two state model for the protein unfolding was considered in the calculation of the ΔG of unfolding (ΔG_U) at each urea concentrations.

Under the assumption of two state model the following two formula can be used for ΔG_U calculation (13, 14):

$$1 = Fu + Fn \quad (2)$$

$$\Delta G_U = \Delta G_U^0 - m[Urea] \quad (3)$$

Where F_n is the fraction of folded protein, ΔG_U^0 is the energy required to unfold the protein at 0 M urea, ΔG_U is the energy required to unfold the protein at increasing urea concentrations, and m is the indicator of the cooperatively of the unfolding. The values for ΔG_U at each urea concentration were determined using the following equation (13, 14):

$$\Delta G_U = -R \times T \times \ln(F_u/F_n) \quad (4)$$

Where R is $8.314 \text{ kcal} \times \text{mol}^{-1} \times \text{K}^{-1}$ and T is 295 K. The ΔG_U° and the m values were then calculated by both linear extrapolation of the transition region via formula 3 and with non-linear estimation with the following formula (13, 14):

$$F_u = 1 / [1 + e^{(\Delta G_0 - m \times [\text{Urea}]/RT)}] \quad (5)$$

2.3 Results and Discussion:

2.3.1 Effects of cAMP on Epac CBD chemical shifts: The chemical shift of each atom in a protein depends upon the micro-magnetic environment around that atom, therefore providing a sensitive probe for structural perturbations upon ligand binding. For this matter, the chemical shift of the amide backbones of the Epac1 CBD-B (Figure 1a&b) were assigned and used to calculate the compounded chemical shift with the following formula (Figure 2a&b) (3):

$$\Delta\delta_{\text{compound}} = ((\Delta\delta^1\text{H})^2 + (\Delta\delta^{15}\text{N}/6.5)^2)^{0.5} \quad (6)$$

where $\Delta\delta^1\text{H}$ and $\Delta\delta^{15}\text{N}$ are the changes between the proton and nitrogen chemical shifts upon cAMP binding respectively.

Figure 2a&b shows the changes upon cAMP docking to the PBC. The most significant perturbations are observed for residues 269-280 corresponding to the PBC helix-loop motif. The large chemical shift variations observed for this motif could be explained by the new magnetic climate introduced by the phospho-ribose moiety of cAMP in the PBC region. In addition, several direct amide backbone to ligand contacts are highly possible between some of the PBC residues, such as G269, A272, and A280, with cAMP according to other known (16) structures of CBDs such as the CBD of LotiK1 channel CBD (PDB: 1VP6) (Chapter 4, Figure 3b). The next significant chemical shift variations

observed are between residues 250-260 corresponding to the $\beta 4$ and $\beta 5$ strands. According to the cAMP bound structures of other known CBDs, such as those of PKA and cyclic nucleotide gated ion channels (CNG) (15, 16), this segment of the domain is believed to be in close proximity to the base of the cyclic nucleotide (Figure 3b).

The plot of the chemical shift against the residue number also revealed perturbations at sites far away from the PBC according to the CBD-B of the Epac2 structure ^{(1O7F)(17)} (Figure 4b). These sites included the $\beta 2$ - $\beta 3$ loop, the NTHB ($\alpha 3$ and $\alpha 4$) and the inner face of the Hinge, and their perturbations upon ligand binding must be due to indirect communication with cAMP due to distance constraints. This suggests that they may play an important role in the relay of cAMP binding information to the RR:CR interface.

The secondary structural response to cAMP was assessed via $^1\text{H}\alpha$, $^{13}\text{C}\alpha$, $^{13}\text{C}(=\text{O})$, $^1\text{H}\beta$ and $^{13}\text{C}\beta$ chemical shifts using PECAN software (7). The overall α/β fold of the CBD in both cAMP free and bound states closely resembles the CBD of the full length Epac2 crystal structure. The $\alpha 1$ helix at the N-terminus of the domain is largely unstructured in the isolated domain (Figure 2c). This helix is stabilized by interacting with the N-terminal DEP domain in the full length protein, hence becomes destabilized in the isolated domain. However, ΔDEP -Epac has been shown to behave similarly to the full length protein and responds in the same manner to the cAMP docking (18). Hence destabilization at the N-terminus of $\alpha 1$ does not disrupt the crucial inhibitory ionic interface between the NTHB and the CR. This isolated CBD construct has also been shown to retain cAMP affinity similar to that of the full length protein (18). Hence this construct can be regarded as the central controlling unit of Epac. Figure 2c shows that the major perturbation upon cAMP binding are focused within the α -subdomain; the Hinge helix which exists in an extended

conformation (D296-K310) is shortened by five residues at its C-terminus, the PBC is stabilized as indicated by higher α -helical probability and the $\alpha 3$ helix within the NTHB is destabilized. The β -subdomain is less sensitive to cAMP in terms of secondary structural perturbations with slight destabilization of $\beta 7$ at the C-terminus of PBC and $\beta 4$ and $\beta 5$ flanking the BBR. Formation of helix-loop-transient helix motif in the Hinge-Lid region upon ligand binding might signify a bending conformation allowing for the interaction between the Lid and the PBC and/or the base of cAMP.

2.3.2 Effects of cAMP on Hydrogen-Deuterium exchange:

HD exchange rates are sensitive probes of the native state ensemble and provide exclusive insight not only on largely populated conformers but also on excited and at least partially unfolded states of proteins, which are invisible in chemical shift mapping (3). The backbone amide proton-deuterium exchange is monitored after the initial D₂O exposure and the intensity of each resonance is plotted against time (sec) and fit in the following equations (3):

$$I_{(t)} = I_0 \times e^{(-K_{ex} \times t)} \quad (7)$$

Where $I_{(t)}$ is the intensity of a resonance after time t , I_0 is the initial intensity of the resonance, K_{ex} is the rate of HD exchange and t is time in sec (3). The measured rates are converted to protection factors (PFs) by division with intrinsic exchange rates computed based on model poly Ala peptide via the software SPHERE (10).

Figure 4a, reveals that upon cAMP binding there is a general enhancement in PFs, which indicates that the protein becomes overall more compact and less exchange competent. Another point evident from the HD profile is the maximum protection ($PF > 6$) for residues of the β -subdomain, especially those of the $\beta 3$, $\beta 4$, $\beta 7$ and $\beta 8$. This suggests that a difference exist in terms of fluctuations and solvent accessibility between the two α -

and β -subdomains with the former having less fluctuations and solvent accessibility than the latter. Selected regions of the protein display high solvent accessibility in the free state, which suggests the presence of significant local unfolding fluctuations, and gain moderate protection ($PF \sim 4$) upon cAMP binding that suggests partial quenching of such local motions. These regions include the Hinge (F300, I303 and I304), the PBC (A272 and R279) and the $\beta 2$ - $\beta 3$ loop (S233, G235 and D236). These regions do not attain the same level of protection observed for the β -strands in the core of the β -subdomain. This observation suggests that in the bound form, the Hinge, the PBC and the $\beta 2$ - $\beta 3$ loop can probe the exchange competent states through a faster mechanism than those of the core β -strands. It is then plausible to argue that these selected regions possess residual local unfolding fluctuations to exchange competent states, in the presence of cAMP, and that the core β -strands can probe these states via slower transient global unfolding fluctuations (3, 18). It is of interest to note that the regions displaying local unfolding fluctuations, the PBC, the Hinge and the $\beta 2$ - $\beta 3$ loop, coincide with regions displaying chemical shift sensitivity toward cAMP (Figure 2a&b). The sensitivity of the PBC, the Hinge, and the $\beta 2$ - $\beta 3$ loop were also independently confirmed at peptide resolution via HD exchange mass spectrometry (19).

2.3.3 Structural interpretation of the effects of cAMP:

2.3.3.1 cAMP docking to the CBD of Epac:

According to cAMP

bound structures of known CBDs, it is expected that the phospho-ribose moiety of the ligand interacts with the N- and C-termini of the PBC (Figure 3b). The proposed interactions not only change the magnetic environment around the PBC but may also result in structural perturbations in this region, such as compacting of the PBC motif, which

could decrease the distance of the two termini of this motif due to the formation of new protein to ligand contacts mediated by N- and C-termini of this motif (Figure 3b and Chapter 4, Figure 3b). This is in align with our chemical shift observations indicating significant changes in the N- and C-termini of the PBC with the simultaneous increase in α -helical probability of the $\alpha 5$ within N-PBC. Also the A280 and R279 at the two ends of the PBC are protected in the cAMP bound state. Such a positioning of cAMP phosphoribose moiety places the base of the cyclic nucleotide in the proximity of $\beta 4$ - $\beta 5$ region, which forms the BBR of the CBD (Figure 3b). The magnetic perturbations introduced via the ring current effect of the adenine base is expected to be sensed by residues in this region. As a matter of fact; higher than average chemical shifts are observed for residues spanning the BBR (Figure 2a&b).

2.3.3.2 Relay of cAMP signal to NTHB: According the cAMP free structure of Epac CBD, there can not be a direct effect from cAMP to the NTHB due to distance constrains (Chapter 1, Figure 2d). There are three lines of evidence of the existence of long range communication networks from the PBC to the NTHB mediated by the $\beta 2$ - $\beta 3$ loop and the Hinge helix. First, we can observe significant chemical shift perturbations in Hinge and the $\beta 2$ - $\beta 3$ loop upon ligand binding. Second, several residues in these regions display remarkable enhancement in protection from D₂O exchange comparable to the ones observed for the PBC. And third, cAMP binding results in C-terminal destabilization of the Hinge helix as judged by secondary chemical shifts and stabilization of the $\beta 2$ at the N-terminus of the $\beta 2$ - $\beta 3$ loop. These two sites are far away from the PBC and BBR and their perturbations can not be explained by direct contact with cAMP. Hence the Hinge helix and the $\beta 2$ - $\beta 3$ loop emerge as two allosteric hot spots within

Epac CBD. Our solution NMR analysis also suggests that the cAMP induced perturbations are spread even beyond the Hinge and the $\beta 2$ - $\beta 3$ loop and also affect the $\alpha 3$ - $\alpha 4$ helices in the NTHB. Significant chemical shift perturbations exist for these two helices in the NTHB and the secondary structural analysis indicate potential perturbations on helical packing since the $\alpha 3$ helix is significantly destabilize upon cAMP binding (Figure 2). Residues at $\alpha 2$ and $\alpha 4$ also display enhancement in protection and reach the level similar to those in the core β -strands ($\beta 3$, $\beta 4$, $\beta 7$ and $\beta 8$). These overall enhancement is in response to the global stabilization of the CBD upon cAMP binding, hence it is expected that they respond concertedly to cAMP global stabilization effects. The immediate chemical shift changes in the $\alpha 3$ - $\alpha 4$ segment may be the direct result of the Hinge movement in response to cAMP binding since this segment faces the Hinge helix in the absence of the cAMP (Figure 3a).

From the discussion above, it is evident that cAMP docking information to the distant PBC and the BBR is reported to the NTHB, which harbors key ionic latch residues for the masking of the CR, via the two newly identified allosteric sites, the Hinge helix and the $\beta 2$ - $\beta 3$ loop. Here we analyze the unliganded Epac2 crystal structure (20) to identify structural interpretations of how these two sites can inform the NTHB of the cAMP binding event.

2.3.3.3 Defining the intra-molecular signaling networks mediated via Hinge

helix:

Previously it has been shown that the PBC and Hinge helix form a hydrophobic bridge via the side chains of F300 at the N-terminus of the Hinge and L273 in the N-terminus of the PBC (20). A close look at the crystal structure of Epac CBD reveals that this contact is part of a bigger interface between these two CBD elements (Figure

4b&c). This hydrophobic bridge is in close proximity to the aliphatic side chain of K297 at the N-terminus of the Hinge helix. K297 makes a hydrogen bond contact with its amino group to the carbonyl backbone of A272 at the N-terminus of PBC. K297 also forms side chain based salt bridge with D276 in the middle of the PBC. This suggests that the interface between Hinge and PBC contains both hydrophobic and polar/ionic contributions. Consistent with these sets of interactions, significant chemical shift variations are observed for F300, A272 and L273. K297 is broaden beyond detection in cAMP bound conformations, but in a smaller construct (Chapter 4, Figure 1a), it display significant chemical shift perturbations. A full appreciation of how these sets of contacts report cAMP binding information to the NTHB is revealed by observation that both F300 and K297 are in contact with critical parts within CBD. F300 forms multiple contacts with the $\alpha 4$ in NTHB via its side chain, which can account for cAMP dependent perturbations in protection factors for this helix, and K297 can communicate with T239 side chain at the $\beta 2$ - $\beta 3$ loop (Figure 4c&d). This signifies the pivotal roles of F300 and K297 that not only connect the PBC and Hinge helix but also relay cAMP signal to distal allosteric sites within the CBD.

2.3.3.4 Defining the intra-molecular signaling networks mediated via $\beta 2$ - $\beta 3$

loop:

A second set of contact can explain the cAMP dependent changes in chemical shift, secondary structure and solvent accessibility observed for residues within the $\beta 2$ - $\beta 3$ loop. This involves two classes of interactions contributed by one of the most conserved residue at the C-terminus of PBC, R279. The first class of interactions is mediated by the backbone of R279. According to unliganded Epac structure, R279 can form two backbone to backbone hydrogen bonds with carbonyl of D236 and amide of

G235 at the middle of the $\beta 2$ - $\beta 3$ loop (Figure 4d). D236 in turn can form hydrogen bond via its amide to the carbonyl of S233, which could results in stabilization of this loop motif. Simultaneous cAMP mediated enhancement in protection from D₂O exchange for these residues indicates that in the presence of the ligand this extended communication network between the PBC and the $\beta 2$ - $\beta 3$ loop is fully nucleated. The role of R279 in radiating the cAMP signal to down stream allosteric sites is not confined to its backbone. The second class of interactions is mediated by the side chain of R279. The guanidinium of this residue forms a hydrogen bond to S240 at the N-terminus of $\beta 3$. S240 resides in a critical position within the β -subdomain. To its N-terminus it is adjacent to T239 which is in contact with Hinge helix via K297 and to its C-terminus it is adjacent to W241, which is in contact with $\alpha 3$ in NTHB, and in close proximity to L293 in $\beta 8$, which is also in contact with $\alpha 3$ (Figure 4d). As a matter of fact we can observe significant changes in secondary structural probability for T239, which is mediated by cAMP (Chapter 4, Figure 2). R279 can also influence the C-terminus of $\beta 2$ via its methylenes interaction with the ring of F232. This can explain the cAMP dependent enhancement in protection factors observed for F232. It is interesting to observe that the transmitted cAMP signal from the PBC to the $\beta 2$ - $\beta 3$ loop can be propagated to the inner strands of the β -barrel, $\beta 3$ and $\beta 8$, which in turn can communicate with $\alpha 3$ helix and results in its observed cAMP mediated destabilization (Figure 4d).

2.4 cAMP recognition and allostery in PKA vs. EPAC:

Similar investigations on CBD-A of PKA-R1 α have also identified the Hinge and the $\beta 2$ - $\beta 3$ loop as two allosteric hot spots within the domain (1-3). These observations suggested that the allosteric response of Epac CBD might reveal a generalized mechanism

for cAMP sensing domains, which is supported by the presence of conserved residues and spatial arrangements within these proteins (21, 22). However two key differences were observed at the level of cAMP recognition and allostery. In the case of Epac the conserved G269 and A280 at the N- and C-terminus of the PBC, which are known to form Hydrogen bond with cAMP in known structures, were not protected in the HD experiments suggesting the lack or weakening of these conserved protein-ligand contacts. This might explain the lower affinity of Epac CBD to cAMP relative to that of the PKA (18). The second difference was the higher overall PF of the NTHB in the Epac compared to the PKA, which suggests a denser contact network resulting in tighter regulation in Epac NTHB, which might compensate for the brief contact with the catalytic region that is much wider in PKA. Also, unlike in the CBD of PKA (3), the cAMP induced global stabilization effects is not only confined to the β -subdomain but also radiates to the $\alpha 2$ and C-terminus of $\alpha 4$ in the α -subdomain (Figure 4a). This is an indication of a stronger inter-subdomain coupling in Epac versus PKA CBDs.

2.4.1 Urea unfolding analysis: In accordance with our HD data, which suggested a more compact network of interactions in the presence of cAMP, we observed a higher resistance of Epac CBD to unfolding with urea in the presence of cAMP (Figure 5). As mentioned previously, the simultaneous protection enhancements for the buried residues that exchange via global unfolding events, indicates less occurrence of such events and hence a more stable structure. This is represented by a higher ΔG of unfolding and a higher C_{50} (concentration of urea necessary for 50% denaturation) for the cAMP bound CBD (Figure 5). The m value is indicative of the degree of cooperativity in the urea induced unfolding (13, 14). The higher the m value the more cooperative the unfolding

even would be (13, 14). Then it is evident that Epac CBD in the presence of cAMP unfolds more cooperatively than in the apo form (Figure 5). It has been suggested that higher cooperativity in unfolding events signify higher couplings between regions of the protein (13, 14). This is parallel to our observation of more compact hydrogen bonding network between different segments of the Epac CBD such as the PBC, the $\beta 2$ - $\beta 3$ loop, the Hinge and the NTHB (Figure 4). It is also interesting to note that the previous unfolding investigations on the CBD-A of PKA-R1 α , suggested a lower m value of unfolding and hence less cooperativity of unfolding in PKA versus Epac CBD (13, 14). From our investigation we have suggested a weaker inter-subdomain coupling between the α - and the β -subdomains in the CBD of PKA versus that of the Epac, which goes in parallel with the observed reduction in the m value for the former.

2.5 Conclusion:

The identification of intra-molecular allosteric network in the Epac CBD resulted in characterization of the Hinge helix and the $\beta 2$ - $\beta 3$ loop as two key allosteric hot spots that can report cAMP binding event to the NTHB, which in the context of the full length protein, makes the auto-inhibitory contact with the catalytic domain (Figure 6). The Hinge helix is responsible for reporting of the events in the N-terminal and central regions of PBC to primary the $\alpha 4$ in NTHB. The $\beta 2$ - $\beta 3$ loop mediates the relay of the C-terminal events of the PBC to mostly $\alpha 3$ and possibly $\alpha 1$ in the NTHB. Significant perturbations in the PBC and the BBR were also consistent, with previous observations in other known CBDs, of the N-terminal capping of PBC with the phospho-ribose moiety of cAMP and the close proximity of the cAMP base with $\beta 4$ and $\beta 5$ (BBR). Also an important difference were observed in the mode of cAMP binding between Epac and PKA with the possible

lack/weakening of conserved protein-ligand contacts mediated by N- and C-termini of the PBC, which could help explain the lower affinity of isolated Epac CBD toward cAMP compared to its PKA counterpart and a weaker inter-subdomain coupling in PKA versus Epac.

One important question raised by this investigation is the nature of the translation of the cAMP perturbations on the ionic latch residues in the NTHB. Although we can observe helical repacking in NTHB by HD exchange and secondary structural probability data, no significant chemical shift perturbations can be observed for residues spanning $\alpha 1$ and $\alpha 2$ that contain the ionic latch residues. In the next Chapter we will investigate the effects of the cAMP binding on the ionic latch region in the NTHB.

2.6 Reference:

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2.7 Figure Captions:

Figure 1) Epac CBD preparation and initial analysis. a) Sequence alignment of CBDs from human Epac1 (Epac1_h), human Epac2 (Epac2_h) and mouse Epac2 (Epac2_m) are shown. Green color indicates conservation of identical residues in all three strains, blue indicates the presence of a homologous functional group of the amino acid side chain and yellow indicates the presence of identical residues in only two strains. Where there are no colors, indicate the lack of conservation. F300 and L273 that form the hydrophobic bridge between PBC and Hinge are highlighted in blue boxes along with ionic latch residues in NTHB. b) The domain organization of Epac1 and Epac2 are shown with the indication of regulatory region (RR) and catalytic region (CR) in dashed boxes. The boundary of the construct studied in this chapter (Epac1 149-318) is shown in brackets. c) The result of ion exchange chromatography on the purified Epac1 149-318 sample is shown and the fractions from the prominent peak are ran on SDS-PAGE with the expected molecular weight of ~19 kDa. d) Left panel: 1D proton spectra of Epac 149-318 with (top) and without (bottom) cAMP. Right panel: overlap ¹⁵N HSQC spectra of Epac 149-318 with (blue) and without (red) cAMP.

Figure 2) Structural respond of Epac 149-318 to cAMP. a) Compounded chemical shift of the amide backbone of residues are plotted against the residue number. Regions that show more than average chemical shift are highlighted in colors and mapped on to the 3D structure of Epac2 CBD (17). The color code is as follows: yellow → Hinge, blue → PBC, green → BBR, purple → β2-β3 loop and red → NTHB. The secondary structures from the crystal structure of Epac2 CBD are indicated with solid lines with positive as α-helix and negative as β-sheet. b) The chemical shifts in respond to cAMP are mapped on to the 3D structure of Epac2 CBD (17) with the same color coding. c) The respond to cAMP in terms of secondary structural probabilities are shown with positive values indicative of α helix and negative that of β sheet. Values close to 0 are indicative of random coil conformation.

Figure 3) Proposed contact and structural respond of Epac CBD to cAMP. a) The structural alignment of CBD-B of Epac2^{(PDB: 1O7F) (17)} in the absence of cAMP (green) with CBD-A of PKA^{(PDB: 1RGS) (15)} in the presence of cAMP (gray). The dashed lines indicated the inward movement of the Hinge/Lid region upon ligand binding. The van der Waals surface of α3 and α4 are shown in red and indicates the close proximity of these NTHB helices to the Hinge/Lid region that can sense the perturbations of Hinge/Lid movements as can be seen in the compounded chemical shift in figure 2a. The structures were made with PYMOL [30] by aligning the β-subdomains of each CBD. b) Alignment of PBC and BBR of Epac2 (2BVY), PKA (1RGS) and CNG (1VP6) CBDs with green, cyan and red colors respectively. The CBDs of PKA and CNG are bound to their respective cAMP molecules that are shown in stick and ball representation. Epac is in the absence of cAMP.

Figure 4) cAMP mediated perturbation in solvent accessibility and proposed intra-molecular cAMP signaling pathway. a) The HD exchange profile of Epac1 CBD (149-318) is shown for both cAMP free (orange) and bound (black) states. The protections factors are plotted on the y-axis in logarithmic scale against the residue number. The PBC,

Hinge and $\beta 2$ - $\beta 3$ loop, which show low to moderate protections, are highlighted with red columns and other regions with maximal protection are highlighted in brown columns. The circles are indication of fast exchange, squares are indication of overlaps and upper and lower arrows are indication of upper and lower limits of protection respectively. The solid lines on the top of the panel are the same as those in figure 2a. b) The intra-molecular cAMP signaling networks mediated by Hinge and $\beta 2$ - $\beta 3$ loop are shown. c) Interactions of PBC and Hinge helix mediated by both hydrophobic (L273-F300) and ionic/polar (K297-A272-D276) contacts. F300 then forms contact with NTHB and L293 at the C-terminus of the $\beta 8$ and parallel to $\beta 3$ can also forms contact with NTHB. d) Interactions between PBC and $\beta 2$ - $\beta 3$ loop are shown contributed mainly by R279 at the C-terminus of PBC. Multiple residues in N-terminus of $\beta 3$ (W241 and I243) then contact the NTHB. Hinge, PBC, $\beta 2$ - $\beta 3$ loop and NTHB are shown in yellow, green, cyan, red colors respectively.

Figure 5) Urea unfolding measurements of Epac1 CBD. a) The fluorescence emission spectra of Epac1 CBD are shown for cAMP bound (left) and free (right) states at various urea concentrations. the proteins were excited at 293nm and the spectrum was recorded from 300-450nm. b) The fractional unfolding is plotted against the urea concentration for both cAMP bound (red) and free (purple) states of Epac1 CBD. The increase in the resistance to denaturation is indicated by an arrow. The U_{50} , which is the concentration of urea necessary for 50% unfolding, are shown by dashed lines. Spectral noise was used in the estimation of error on the fractional unfolding curves. The inset is the linear extrapolation of the transition region that is used in the linear based calculation. c) The calculated value for the ΔG° , m are represented with linear and non-linear based method for estimation. C_{50} is the concentration of urea required for 50% denaturation.

Figure 6) Schematic representation of cAMP allosteric network in Epac CBD.

cAMP binding to the PBC is reported indirectly to the inhibitory ionic interface between RR and CR via Hinge helix and the $\beta 2$ - $\beta 3$ loop. This reported message then can cause perturbations in helical packing and other yet uncharacterized effects that can results in the weakening of the interface, which can assist the rigid body rotation of the RR away from the CR initiated by Hinge rotation upon cAMP binding and hence allow for Rap binding.

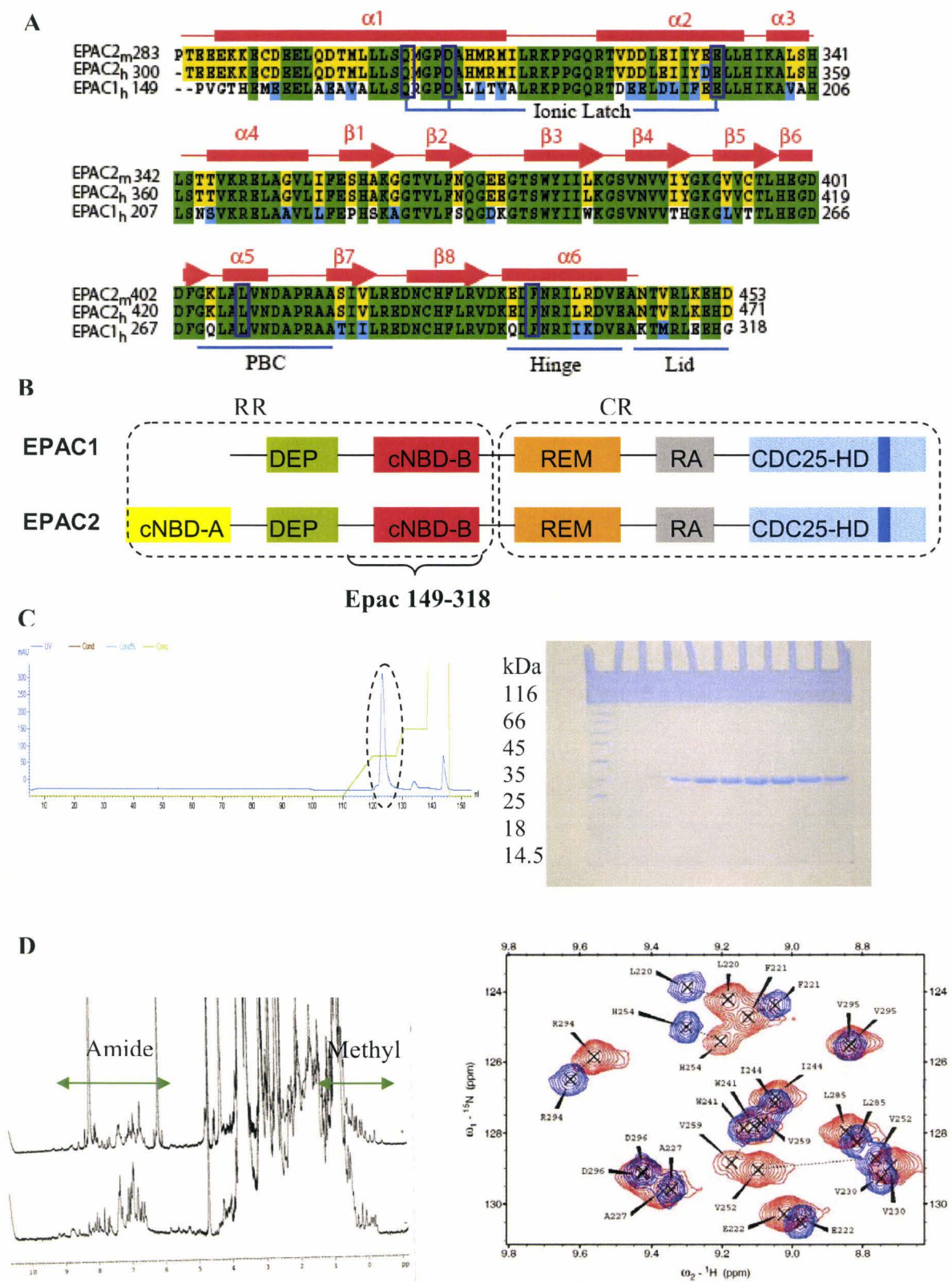


Figure 1

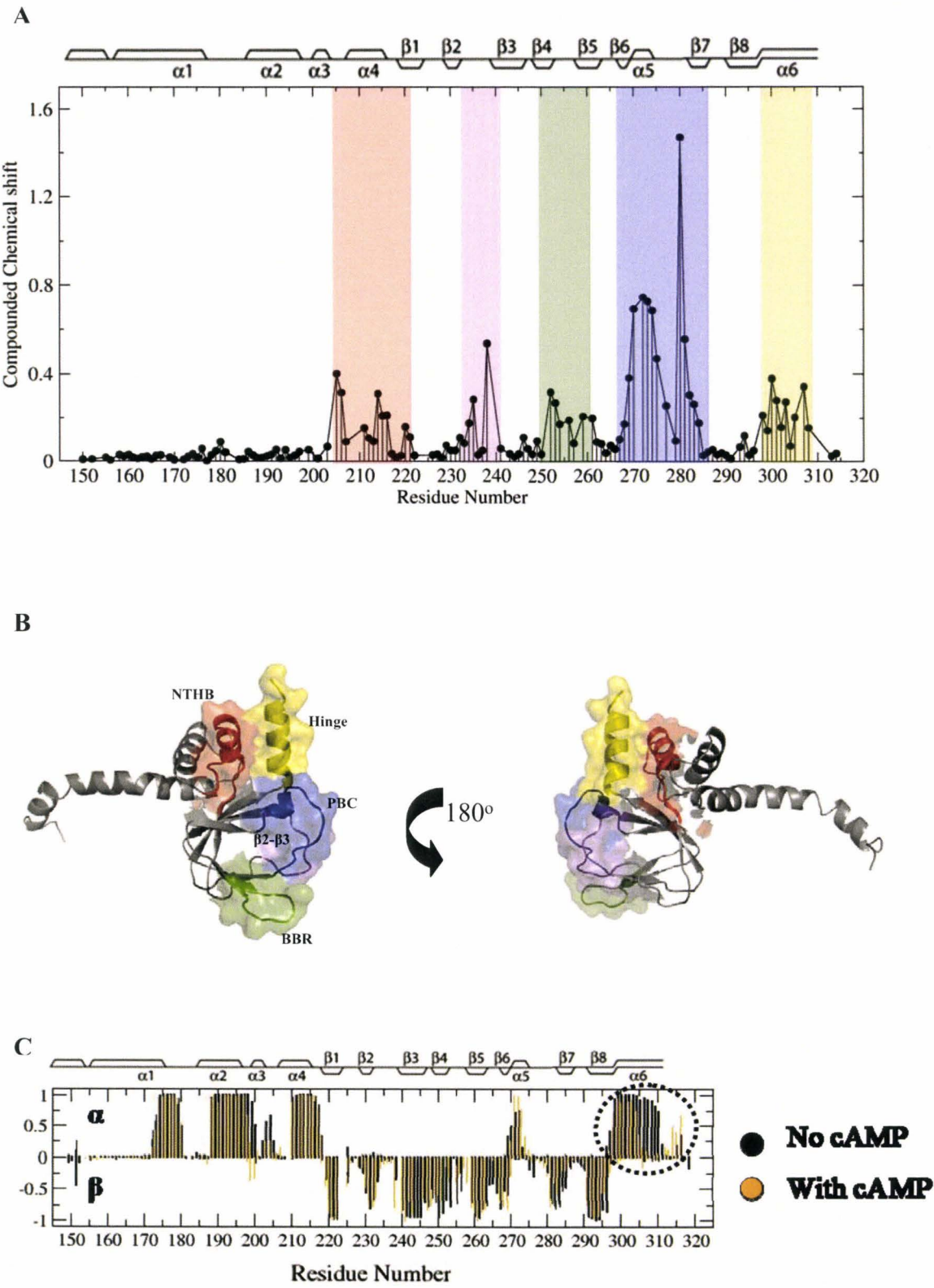


Figure 2

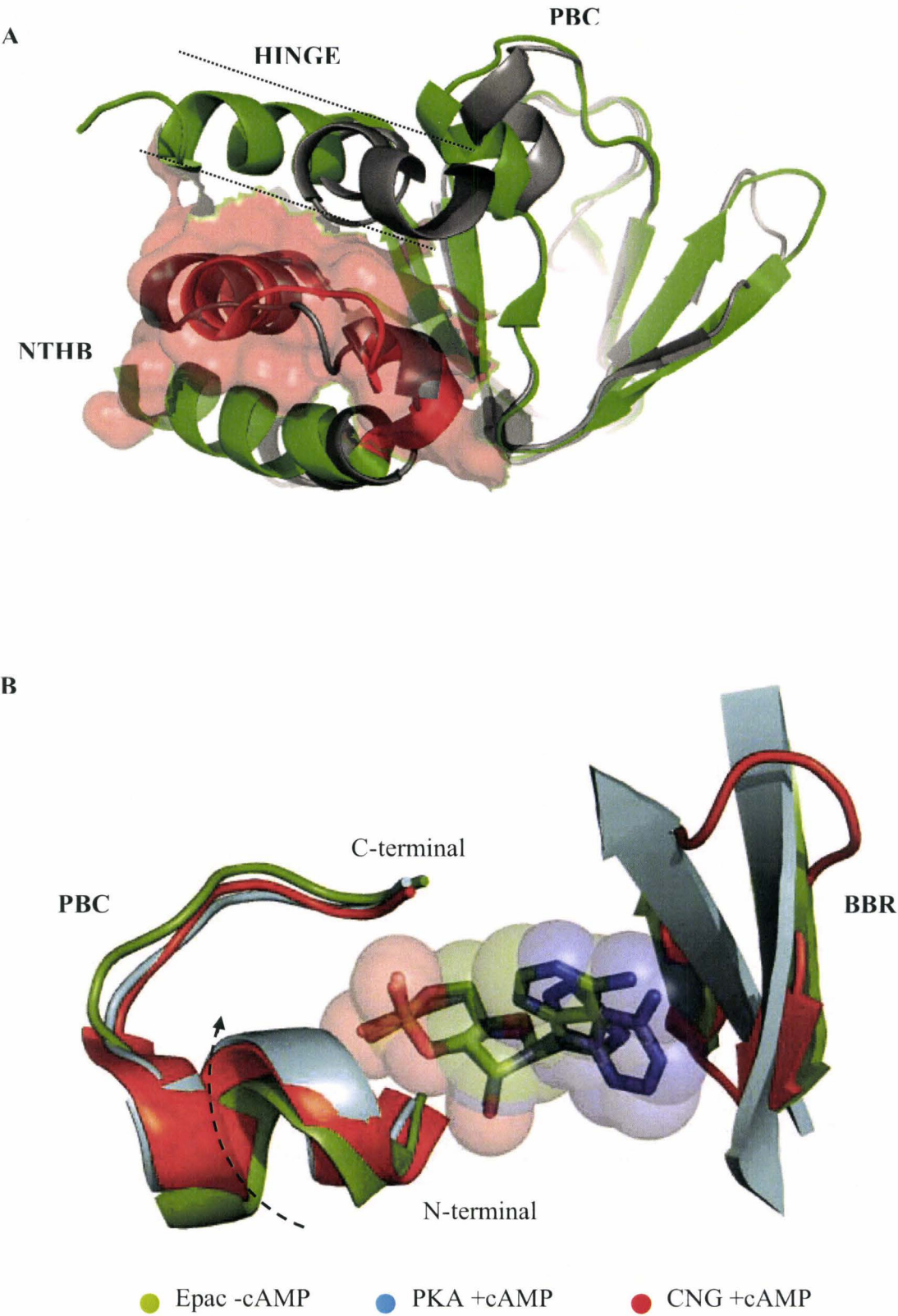
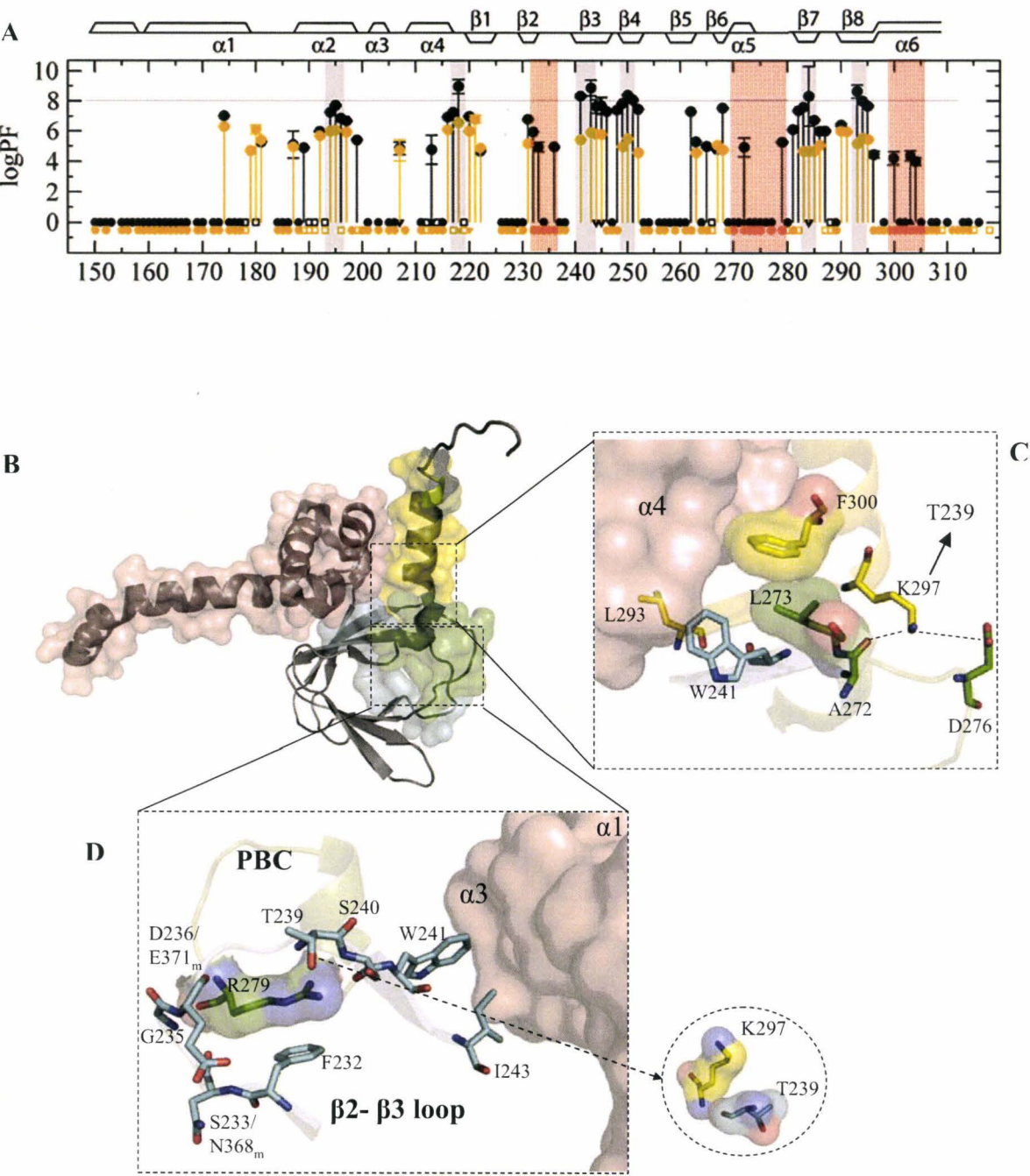


Figure 3



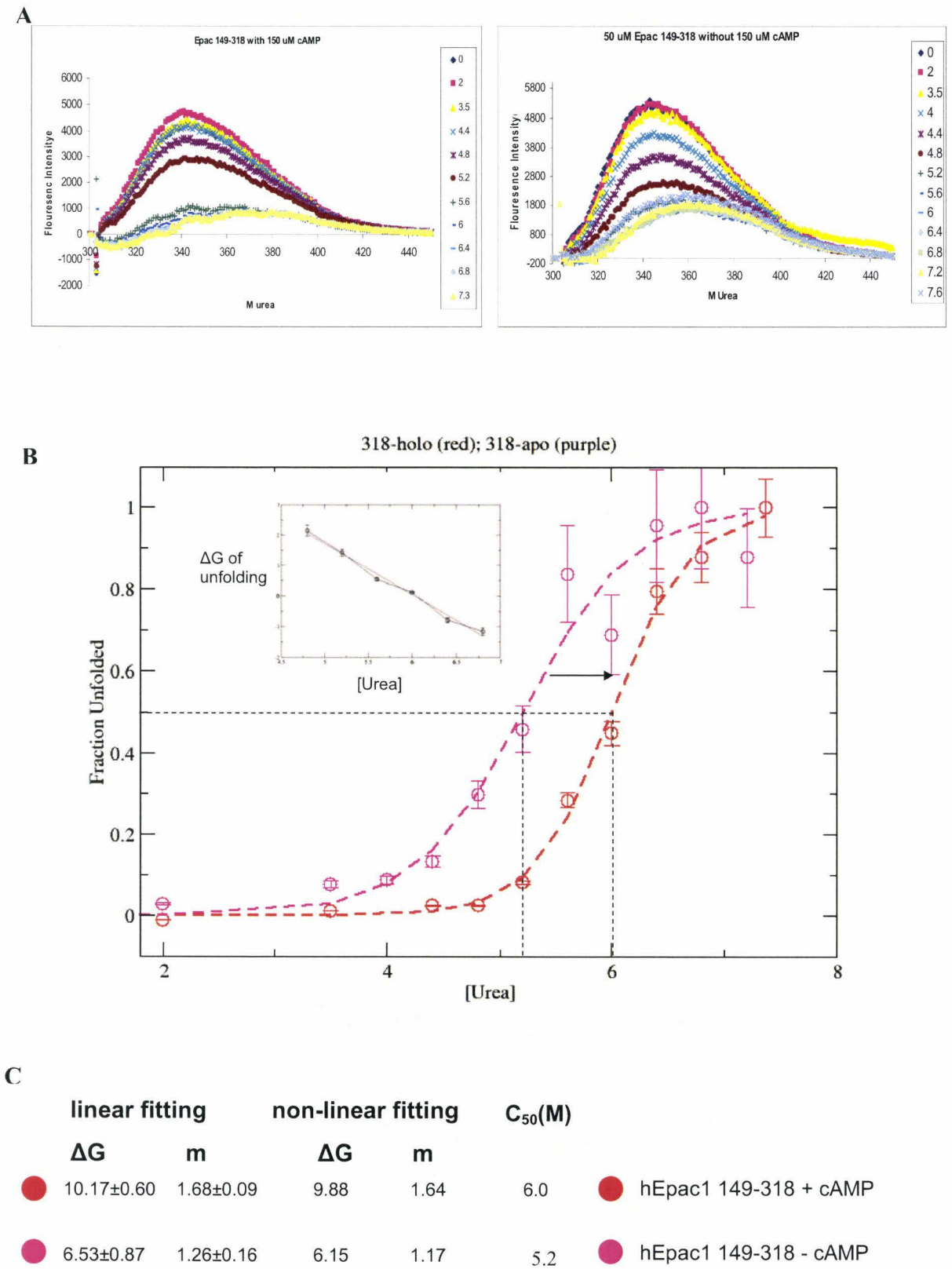


Figure 5

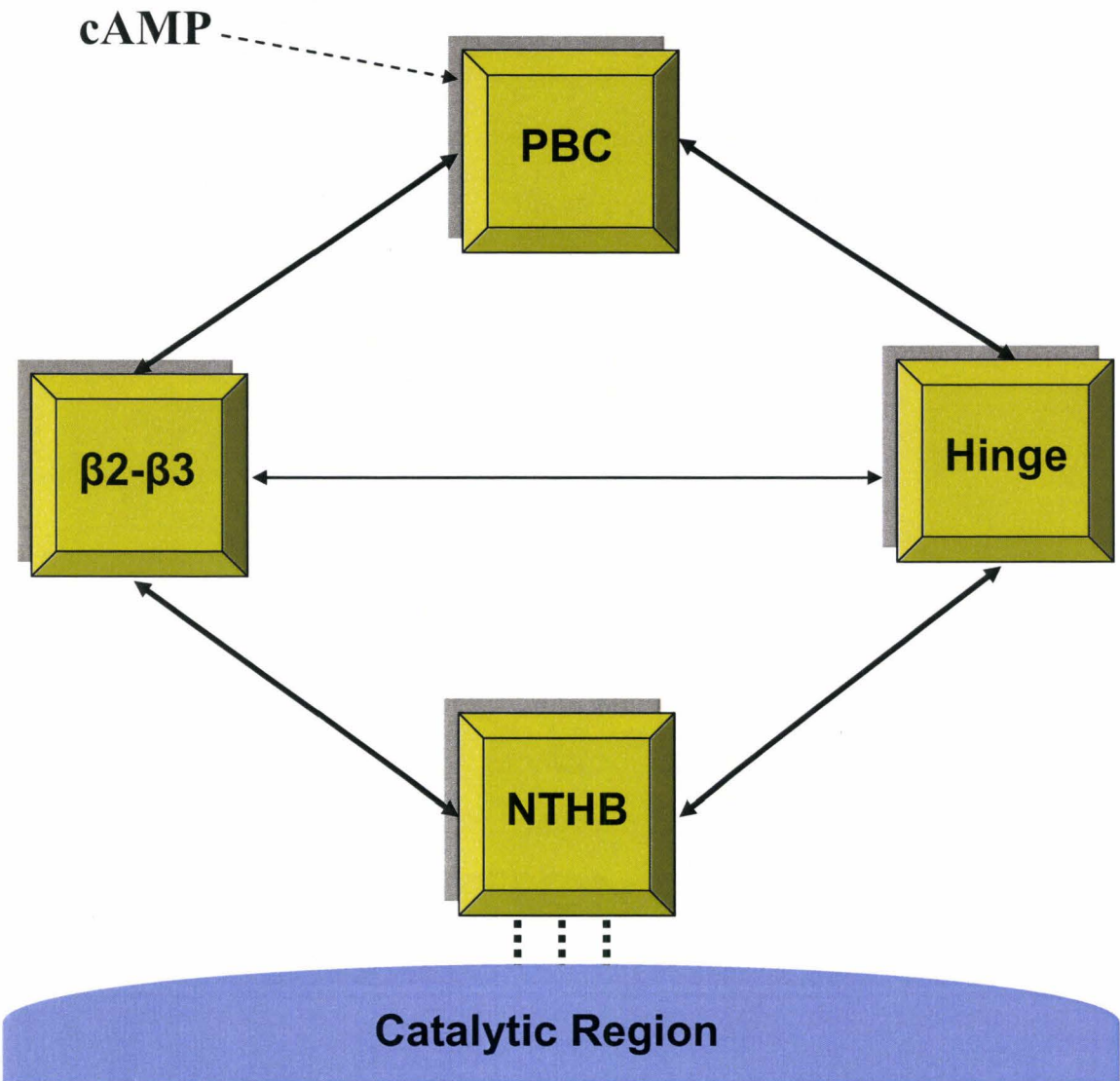


Figure 6

3.1 Dynamic Driven Allostery:

The crystal structures of known CBDs in the presence and absence of cAMP, have hinted to the cAMP mediated hinge-like rotation of a helix C-terminal to the β -subdomain as a key to the Epac activation process. In all the CBDs solved to date the primary cAMP induced structural changes are mapped to the C-terminal Hinge helix. Mutational investigations have suggested that upon docking of the ligand to its binding sites in the PBC and the BBR, the orientation of the former allows for the Hinge helix to move toward the PBC and this movement is hypothesized to change the orientation of the regulatory region (RR) and catalytic region (CR) with respect to each other, opening the periphery of the Rap binding site for nucleotide exchange (1). As mentioned previously in Chapter 1 and 2, the mechanism of Epac activation has been presented in terms of an equilibrium between the active (open conformation) and inactive (close conformation) states and the cAMP mediated Hinge rotation is proposed to shift the equilibrium toward the open conformation. However mutational data have also mapped another site that is important in the regulation of Epac activation equilibrium. This site is within the first two α helices of the NTHB and is termed ionic latch, which forms the ionic interface between RR:CR. Mutations in this region has been shown to shift the equilibrium toward the active state (2).

At the moment, it is not well understood if the Hinge rotation provides sufficient energy for the disruption of the RR:CR ionic interface or that there are additional perturbations in the ionic latch region, which weakens this interface for the re-orientation of the two catalytic and regulatory halves of the Epac. Independent evidences, such as mass-spectroscopic HD exchange (3) and chemical shift mapping (Figure 1a), have suggested the lack of significant structural perturbations in the ionic latch region upon cAMP binding. Hence, we hypothesized and investigated the role of the dynamics in the

cAMP-mediated regulation of the ionic latch region. Our investigations suggested that dynamics is a key effector of the cAMP mediated weakening of the ionic latch via increase in entropic penalty for the RR:CR interface maintenance and establishment of a negative cooperatively between cAMP:PBC and RR:CR contacts.

3.2 Results and Discussion:

3.2.1 Lack of major cAMP-mediated structural perturbations in the NTHB: Our

^1H , ^{15}N chemical shift data and secondary structural probability analysis, consistently indicated the absence of major cAMP-mediated structural perturbations in the region spanning the ionic latch residues in the NTHB ($\alpha 1$ and $\alpha 2$). The magnitude of chemical shift change in IL region is below average and comparable to residues in the $\beta 8$ strand of the β -subdomain, which are known to be largely insensitive to cAMP-mediated structural perturbations in other known CBDs (Figure 1). Consistent with these observations, no secondary structural variation is observed for the $\alpha 1$ and the $\alpha 2$ helices upon ligand binding. Hence, both NH compounded chemical shifts and secondary structures consistently suggest that the region spanning the ionic interface between RR:CR is not affected by cAMP in terms of structural change, consistent with previous observation of the lack of major cAMP mediated effects on the hydrogen-deuterium exchange pattern of ionic latch region in the $\Delta\text{DEP-Epac1}$ (3). The changes observed for residues C-terminus to the $\alpha 2$ are consistent with the model discussed in the previous chapter with cAMP docking to the PBC and the BBR resulting in the relay of binding perturbations via the $\beta 2$ - $\beta 3$ loop and the Hinge helix to the NTHB. The $\alpha 3$ - $\alpha 4$ segment is then forms the interface between the Hinge helix and the NTHB and is directly perturbed by the Hinge movement (Chapter 2, Figure 3a).

3.2.2 ^{15}N relaxation: Molecular motions within proteins results in constant change in local magnetic field. These fluctuations in local magnetic environment can provide a path for transfer of magnetization between different nuclei, provided that their frequencies of motion are similar. In a static magnetic field, B^0 , all spins (magnetically active nuclei such as ^1H or ^{15}N) possess a net macroscopic magnetization (M_z) parallel and in the direction of the B^0 field. In this state the spin is in thermal equilibrium. Upon the application of a transient 90° pulse on the spin (with respect to Z axis), the net magnetization is placed in the xy plane. Relaxation is then defined as the time required for the net magnetization to reach the thermal equilibrium. There are two primary mechanisms that contribute to relaxation in proteins; dipole-dipole interactions (DD) and chemical shift anisotropy (CSA) (4, 5). DD is field independent and is important in molecules that contain high percentage of proton, since ^1H has a high gyromagnetic ratio (dipole magnetic moment/angular momentum). CSA is field dependent relaxation mechanism and arise due to the presence of anisotropic electron density around a magnetically active nucleus. Variations in local magnetic field then provide a possible relaxation pathway. NMR mediated protein relaxation measurement typically use uniformly labeled ^{15}N proteins for amide backbone dynamics measurement. This will help to simplify motional contributions to relaxations since there would be minimal contribution from cross-relaxations between different magnetically active nuclei, such as $^{13}\text{C}_\alpha$ - $^{13}\text{C}_\beta$, which can complicate dynamic interpretations.

Immediately after the initial 90° pulse, the net magnetization is in the xy plane. As time elapses, the Z magnetization builds up as the energy of the spin is transferred to the surroundings or the lattice. The time requires for the build up of Z magnetization after the

initial 90° pulse is termed T_1 and is called spin-lattice or longitudinal relaxation. T_1 is an in-adiabatic process that results in the release of energy to the medium (4). The inverse of T_1 is R_1 and is the rate of longitudinal relaxation. In proteins this relaxation parameter tends to probe for fast ps-ns motions. On the other hand, T_2 relaxation is the adiabatic process of spin-spin magnetization transfer, which results in de-phasing of xy magnetization (4). R_2 is the spin-spin or transverse relaxation rate. R_1 is usually a slower rate than R_2 due to the presence of local magnetic fluctuations which will de-phase the xy magnetization. R_2 is sensitive to both fast ps-ns and slower μ s-ms dynamics within the proteins.

Two additional methods for probing protein backbone dynamics are also widely used, $\{^1\text{H}\}-\{^{15}\text{N}\}$ steady state NOE and NMR relaxation dispersion (NMRD), which probes for fast local ps-ns and slow ms motions respectively (4, 5). In steady state NOE experiment, the dynamics of NH bond vector is probed via measuring the extent of saturation/magnetization transfer between the two nuclei. NMRD probes for slow ms exchange by application of varying re-focusing pulses (4). If a spin exist in two chemically different environments in which the rate of exchange between them is in $\sim$ ms time scale, then the signal from the spin will broaden and tend to average out the two different chemical environments. It is possible to suppress this chemical exchange by application of high number of refocusing pulses in a fixed time interval, which is the basis of Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion experiment (5). Hence, if a resonance possesses slow ms exchange between two chemically different states, there would exist a line width dependence of the resonance on the number of refocusing pulses in the CPMG

experiment. The higher the number of pulse the less line broadening will occur and the sharper/more intense signal and vice versa.

3.2.3 Strategy toward the assessment of dynamics in Epac1 CBD: The

dynamics in ps-ns and μ s-ms time scales, of Epac1 (149-318) were examined in both the apo and cAMP bound states via the measurement of ^{15}N R_1 and R_2 relaxation rates and of $\{^1\text{H}\}-\{^{15}\text{N}\}$ NOEs (Figure 2). The latter is relatively insensitive to the contributions of the global tumbling of the protein to the relaxation rates. According to the HD and secondary chemical shift data, the flexibility of the N-terminus of CBD, specifically the N-terminus of $\alpha 1$, will hamper the accurate description of a diffusional tensor that can describe the overall tumbling of the molecule as a well-defined single diffusion tensor (6). Hence in our analysis of dynamic data we used primarily the methods that do not rely upon an accurate assumption of the overall tumbling of the protein. Hence we used reduced spectral density mapping approach in the analysis of our relaxation data (Figure 3). Possible contributions from the diffusional anisotropy of the overall tumbling were taken into consideration via the evaluation of the R_1R_2 product (Figure 2) that is insensitive to the anisotropy of the diffusion tensor (6), and via hydrodynamic simulations of the ^{15}N relaxation rates (red lines in Figure 2) and the corresponding spectral densities (red lines in Figure 3).

Due to the unstructured nature of the N-terminus of the $\alpha 1$, the hydrodynamic simulation of the relaxation rates was carried out with three levels of N-terminal truncation (truncation from 149, 161, and 173). The most accurate modeling was achieved by truncation at residue 161, however, all three simulations are represented to provide a conservative range of variability in relaxation rates and spectral density contributed by the overall tumbling (6). In addition, dynamics in the slow ms regime was probed via NMR

dispersion (NMRD) measurements that are also insensitive to the overall tumbling of the protein and are reported in figure 4. Hence, the analysis of the dynamics of the Epac1 CBD is to the large extend structure-independent since it depends on a combination of HN-NOE, R_1R_2 , reduced spectral densities and NMR dispersion measurements. $J(\omega_H + \omega_N)$ and HN-NOEs probe for fast local ps-ns dynamics, whereas $J(0)$, R_1R_2 and the NMRD can sense the dynamics in the μ s-ms time scales.

3.2.4 The dynamics of the Epac1 CBD in the absence of the cAMP: The

dynamics of apo Epac1 CBD was assessed in both ps-ns and μ s-ms time scales. For motions in ps-ns range, the most significant features of the CBD are the high flexibility of the N-terminus of $\alpha 1$ helix, as revealed by the high $J(\omega_H + \omega_N)$ and low NOE values for residues in this region (Figure 2&3). This is also an indication of why the hydrodynamic based modeling of the overall tumbling of the protein is more accurately agree with the dynamic data for the N-terminally truncated molecule. There are two more sites within this CBD that exhibits significant motions in ps-ns time scale and they are mapped to the $\beta 2$ - $\beta 3$ loop (D236) and residues in the Lid region. As can be seen from the NOE data D236 possess NOE value below 0.5, indicating the presence of local ps-ns fluctuations. This is in agreement with the hydrogen-hydrogen (HH) data (7) that shows the NH backbone of this residue possess fast exchange with H_2O despite being involved in backbone to backbone contact with other residues. The flexibility of the residues in the Lid region are also corroborated with the fast HD exchange and PECAN data indicating the un-structured nature of Lid in the absence of cAMP (Chapter 2, Figures 4a and 2c).

The dynamics of Epac1 CBD in the absence of the ligand are not confined to ps-ns motions. $J(0)$, R_2 , R_1R_2 and NMRD data, collectively indicate the presence of significant

μ s-ms and slow ms dynamics within the CBD in the apo state. Several higher than average $\Delta R_{2,\text{eff}}$ values are focused in the C-terminus of $\alpha 1$, the $\alpha 4$ - $\beta 1$ and $\beta 2$ - $\beta 3$ regions, A277 in the PBC and the Hinge helix that signifies the presence of slow ms motions at these sites (Figure 4). In addition to these observations, $\alpha 3$ in the NTHB possesses significantly higher R_2 rates and $J(0)$ values compared to those predicted to derive solely from the overall tumbling of the protein (Figure 2&3). This is also confirmed with the higher R_1R_2 rates, which are approximately insensitive to the contributions from the overall tumbling (6), for residues in the $\alpha 2$ - $\alpha 3$ segment (E197, H200, I201 and A203). Another note from the dynamics in this time scale is the significant line broadening effects for residues within the BBR and the PBC in the absence of the cAMP (Figure 2c). Line broadening is the result of slow ms chemical exchange between magnetically different environments with different chemical shifts (6). Hence this also indicates the presence of slow ms motions in these two regions that are escaped from detection in the NMRD experiment. This is also in accord with our observation in the previous Chapter that the PBC and the BBR exhibit lack of protection from D_2O exchange in the absence of cAMP (Chapter 2, Figure 4a). Hence, as can be seen from the 3D dynamic map (Figure 5) of Epac1 CBD in the absence of ligand, multi time scale dynamics in ps-ns, μ s-ms and slow ms are scattered through both the α - and β -subdomains.

3.2.5 Functional relevance of the apo-state dynamics:

The multi-time

scale dynamics in the absence of the ligand are mapped in to the 3D structure (Figure 5). It is worth to note that this is the first high resolution dynamic assessment of a eukaryotic CBD in the apo state since previous attempts on other CBDs failed due to low stability of this state (8). Hot spots of dynamics are mapped in several patches that span regions of this

molecule that are known to be functional in ligand recognition and allostery. For example, both the flanking regions of the PBC and the spatially adjacent $\beta 2$ - $\beta 3$ loop of the β -subdomain possess high motions in μ s-slow ms and ps-ns time scales. This is also true in the case of the α -subdomain, which several patches with higher than average ps-ns and slow ms motions are observed for Hinge helix and the $\alpha 1$ - $\alpha 2$ region containing the ionic latch. The $\beta 1$ strand in close proximity to $\alpha 1$, and $\alpha 4$ in close proximity to Hinge helix also possess higher than average dynamics in the apo state. The high degree of co-localization of functionally relevant regions with patches of higher than average dynamics in the absence of the cAMP, might be explained by the presence of equilibrium between active and inactive states that has been previously reported (1) via bio assays with the Epac protein. It is also important to stress that the dynamics of the apo state will be helpful in elucidation of the effects of cAMP on entropy, since this state can be used as a reference point for comparison. The structure of the full length Epac reveals significant increase in the B-factors for residues spanning the N-terminus of the $\alpha 1$ (1). This suggest that the presence of significant ps-ns dynamics in this region (Figure 5) might, at least to some extent, be indicative of an intrinsic property of this helix and not merely due to the truncation effects.

3.2.6 cAMP mediated dynamic modulation of Epac1 CBD:

Although in the apo state, several scattered loci in α - and β -subdomains display multi-time scale dynamics, cAMP affects these two subdomains in a remarkably different ways. While docking of the ligand to the PBC results in the quenching of slow ms dynamics in β -subdomain, as evident from the decrease in $\Delta R_{2,eff}$ values of F232, I243 and I244 within the $\beta 2$ - $\beta 3$ regions and A277 in the PBC helix-loop motif, there are simultaneous dynamic enhancements in

the α -subdomain for residues spanning the ionic latch region in $\alpha 1$ and $\alpha 2$ helices (such as, V177, L179, K181 and I194) and the C-terminus of the Hinge helix, K305 and E308 (Figure 2-4). From line broadening analysis, it is evident that $\sim$ ms dynamics of the PBC and the BBR are also quenched upon docking of cAMP due to the narrower line width in the signals from these two regions (Figure 2c). In the case of μ s-ms time range, we can observe F232 and A281 in the $\beta 2$ - $\beta 3$ loop and the $\beta 7$ strand, respectively, quenched in dynamics while E197 in $\alpha 2$ and I201 and A203 in $\alpha 3$ helices of the NTHB enhanced in μ s-ms. The dynamic elevation in $\alpha 3$ and the C-terminus of the Hinge helix are also independently confirmed by secondary chemical shift data, which shows destabilization of these two helices upon cAMP binding (Chapter 2, Figure 2c). This inverse dynamic response toward the ligand binding does not limit to these two time scales and is also seen in the fast local ps-ns range. From the steady state NOE and $J(\omega_H + \omega_N)$ data, it is evident that D236 in the absence of cAMP possess fast local motions with NOE value ≤ 0.5 , while in the bound state the NOE is ~ 0.9 and within error range of NOE values predicted from global tumbling of the protein (Figure 2e). This is a significant quenching of dynamics and is also confirmed by remarkable enhancement in the protection from D_2O exchange (Chapter 2, Figure 4a). Simultaneously several loci scattered within NTHB, enhance in ps-ns motion such as R169, D172 and A173 in the $\alpha 1$, I201 in the $\alpha 3$ and A216 in the $\alpha 4$. Several ps-ns enhancement in C-terminus of Hinge and the adjacent Lid region is also observed for K305 and R313 consistent with C-terminal Hinge destabilization from the secondary chemical shift data.

3.2.7 Functional relevance of cAMP-mediated control of dynamics: Effects of the cAMP on dynamics of Epac1 CBD are mapped on to the 3D structure (Figure 6). From

the first place it is evident that the cAMP mediated perturbations in dynamic landscape are not confined to the PBC and mapped well to the critical functional sites. Quenching of dynamics in the PBC is the results of stabilizing effects conferred via docking of cAMP and the formation of inter-molecular contacts. This stabilizing effect is extended to the spatially coupled $\beta 2$ - $\beta 3$ loop. In the previous Chapter, it was shown that this highly fluctuating loop, in the absence of ligand, is stabilized by several backbone to backbone interactions involving G235 amide and S233 carbonyl functional group. This nucleation of the β -turn, then will stabilize F232 at the C-terminus of $\beta 2$, which is highly distorted in the apo Epac CBD. This cluster of cold patches (quenched dynamics) are in close proximity to R279 and G238 in PBC and $\beta 2$ - $\beta 3$ loop respectively (Figure 6a). Extensive evolutionary analysis of genomic CBDs have suggested that these two residues have co-evolved to both coordinated cAMP binding through R279 and relaying the binding event to down stream functional sites via G238 (9). The importance of G238 can be further highlighted with its high chemical shift sensitivity toward cAMP, while being far away from the ligand binding site, and sequence alignment of Epac and PKA-R1 α CBDs, identify G169 as the corresponding residue in the PKA that has been shown to be critical in cAMP mediated activation of PKA via genomic screening (10). Hence, these observations highlight the functional importance of the allosteric modulations in dynamics in these regions and may suggest that the CH- π interaction between the side chain of R279 and the C α backbone of the G238 (11) could account for the observed dynamic based allosteric coupling between these two functional regions, the PBC and the $\beta 2$ - $\beta 3$ loop.

The mix pattern of entropic behavior of the $\alpha 4$ and the Hinge helix is fully consistent with the model of cAMP mediated Hinge movement toward the PBC in the core of the β -

subdomain. Comparison of Epac and PKA CBDs reveals that according to the cAMP mediated Hinge rotation model, N301 and R302 in the middle of Hinge helix come in close proximity to V211 in $\alpha 4$ helix and can explain the simultaneous dynamic quenching in slow ms range observed for these three residues (Figure 6c). On the other hand residues at the C-terminus of Hinge helix, such as K305 and E308, and at the C-terminus of $\alpha 4$, V218, enhance in dynamics. According to the Hinge rotation model, the N-terminus of Hinge helix is moved away from the C-terminus of $\alpha 4$ helix upon ligand binding and simultaneously K305 and E308 are displaced away from the $\alpha 4$ helix. According to secondary chemical shift data (Chapter2, Figure 2c), K305 marks the point of cAMP mediated Hinge destabilization and is also the only residue displaying significant cAMP mediated ps-ns dynamics enhancement (Figure 2e and 3c). Considering that K305 and E308 are in backbone to backbone hydrogen bond contact, the decreased α -helical probability for 305-309 segment marks the enhance flexibility upon cAMP binding in this region, which connects Epac CBD to the CR and is proposed to be crucial in the coordination of the relative orientation between the regulatory and catalytic halves of the Epac protein, which control the nucleotide exchange activity of Epac.

NTHB, another key site that plays an important role in the Epac activation by forming the ionic RR:CR interface and sterically blocking the access of CR to the Rap protein, is also affected by cAMP mediated modulation of the dynamic landscape. The $\alpha 1$ and $\alpha 2$ helices containing the ionic latch residues; Q169, D172 and E197, are all co-localized with loci that display multi-time scale dynamic enhancement upon cAMP binding (Figure 6b). Taken together with the fact that $\alpha 1$ - $\alpha 2$ segment is insensitive to cAMP in terms of structural change (Figure 1); dynamics emerge as a key carrier of cAMP action in the ionic

latch region. Given the importance of the ionic latch in the control of the Epac activation equilibrium, as recently confirmed by mutations in this region and its effect on increase in $K_{\max}$ (1), it will be critical to recognize the entropic contribution in Epac allosteric regulation. After cAMP docking to the PBC, the dynamics in the ionic latch region increase and the entropic cost of maintaining the ionic interface between the RR:CR increases, hence facilitating the release of this inhibitory contact that is presumably initiated by the cAMP mediated Hinge movement (Figure 7). Hence a dynamic mediated negative cooperativity exist between the RR:CR and PBC:cAMP interfaces that is critical in the Epac activation process.

Two important notes deserve to be mentioned in this section. First, the ionic latch residue at the N-terminus of $\alpha 1$, R169, might be argued to be affected by N-terminal truncation artifacts. However by looking at the B-factors of the CBD in the context of the full length protein, it is evident that this region possesses intrinsic dynamic higher than one standard deviation of the CBD average B-factors (1). Hence the high flexibility of the segment N-terminus to $\alpha 1$ at least in part reflects the intrinsic property of the proteins in this region. Second, the cAMP mediated dynamic perturbations are expected to be even more significant in the context of the full length protein due to the formation of the numerous stabilizing interactions to the NTHB, specially $\alpha 1$ - $\alpha 2$, contributed via the DEP and the catalytic domain that will further quench the dynamics in the apo state.

3.2.8 Dynamic allostery in Epac vs. PKA:

Similar studies on the CBD-A of PKAR1 α have suggested similar patten of dynamic quenching in the β -subdomain and enhancement in the α -subdomain (8). The study on the apo PKA was hampered due to low stability of this state and was circumvented by the use of a PKA antagonist Rp-cAMPS in

which the exocyclic equatorial oxygen of the phospho-ribose moiety is replaced with a phosphate atom. Both systems displayed increased in flexibility in the PBC and the $\beta 2$ - $\beta 3$ loop in the absence of cAMP and bound to the antagonist, in the case of PKA, hinting to the critical dynamic role in allostery between these two sites in the relay of cAMP signal to the distal allosteric sites. Due to the poor sequence conservation in the NTHB between the two domains, it is challenging to investigate the dynamic effects on this site. However in both CBDs, the helix corresponding to $\alpha 3$ in Epac, display significantly higher dynamics in the cAMP bound form. Given that the corresponding helix in PKA CBD-A is the sites of critical contacts between the regulatory and the catalytic subunits (11), the effects of dynamics in the activation of CBD associated proteins, investigated in this Chapter in Epac CBD, might reveals a more generalized mechanism of allostery. Recent investigations of the effect of Rp-cAMPS on Epac1 CBD, has confirmed the similar response of both Epac and PKA to cAMP in terms of dynamics (6). In the case of PKA, comparison of the cAMP bound and C-subunit bound regulatory region, confirms that the presence of cAMP results in destabilizations of salt bridges that are responsible to the stability of the NTHB of the CBD-B of PKA (12, 13).

3.3 Conclusion:

In this study, we investigated the multi-time scale dynamics of human Epac1 CBD in the presence and absence of cAMP. In the absence of the ligand, several patches with dynamics in ps-ns and μ s-slow ms range were mapped well to the sites known to be critical in the mechanism of Epac activation. This observation was well in accord with the hypothesis of Epac existing in equilibrium between the active and inactive states that is shifted to the active side via cAMP, as suggested by previous mutational investigations (1).

Binding of cAMP to the PBC resulted in the quenching of the dynamics in the β -subdomain and simultaneous enhancement in the dynamics of the α -subdomain. The positive cooperatively in dynamics quenching, seen for the PBC and the $\beta 2$ - $\beta 3$ region upon cAMP binding, further highlights the importance of the $\beta 2$ - $\beta 3$ loop in the relay of cAMP binding information to the distal allosteric sites. This is in full agreement with the sequence based search of genomic CBDs that suggests PBC and the $\beta 2$ - $\beta 3$ loop have co-evolved (9). The contacts between the ionic latch and the catalytic region on the other hand, were negatively cooperated with the contacts of PBC and the cAMP. While the PBC were rigidified upon ligand docking, several patches with dynamics in ps-ns and μ s-slow ms range were observed and co-localized with the ionic latch region in NTHB. Considering the lack of significant cAMP mediated structural perturbations in the ionic latch region, dynamics emerge as a key factor in the weakening of the salt bridges between the RR:CR interface upon cAMP binding. Together, these data with the observation of the dynamic enhancement in the C-terminus of the Hinge helix that defines the relative orientation of the RR and CR, propose a modified model of the mechanism of Epac activation that considers the entropic contributions in the Epac activation process (Figure 7). According to this model, binding of cAMP to the PBC results in the inward rotation of the Hinge helix toward the PBC, due to the new orientation of the PBC motif. Simultaneously, the dynamics of the β -barrel decrease and that of the α -subdomain increase, which maps to the C-terminus of the Hinge and the NTHB containing the ionic latch region. The flexibility of the C-terminus of the Hinge and the entropic mediated weakening the ionic RR:CR interface, help the re-orientation of the two regulatory and the catalytic halves of Epac that opens up the periphery of the Rap binding site for nucleotide exchange.

3.4 References:

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3.5 Figure Captions:

Figure 1) Lack of significant cAMP mediated structural perturbations in ionic latch region. a) comparison of the selected resonances from ionic latch region (G170 from $\alpha 1$ and T187 from $\alpha 2$) with structurally invariant regions of the β -subdomain (N289 from $\beta 8$ and V249 from $\beta 4$). b) Overlap of the structures of the β -subdomains from different CBDs (PDB codes; PKARI α :cAMP $\rightarrow$ 1RGS, PKARI α :CR $\rightarrow$ 2QCS, Epac2 $\rightarrow$ 1O7F, CNG:cAMP $\rightarrow$ 1VP6, CNG $\rightarrow$ 1U12). The inset zooms in the $\beta 8$ strands.

Figure 2) Relaxation profile of Epac1 CBD (149-318) with (black) and without (orange) cAMP. a) Transverse/spin-spin relaxation rates R_2 b) Longitudinal/spin-lattice relaxation rates R_1 . c) Intensity (I) plot of the resonance in the 2D HSQC spectrum. I_{cAMP}/I_{+cAMP} were plotted against the residue number. Low values indicate the presence of $\sim$ ms motions in the apo state and vice versa. d) Products of R_1 and R_2 that are insensitive to diffusional anisotropy from the over tumbling. e) Steady state NOE values plotted against the residue number. In all cases the increase (decrease) in dynamics is highlighted in red (blue) columns. The red lines represent the hydrodynamic simulation via the HYDRONMR (6) software that compute the contributions of the overall tumbling motion to the dynamic rates. The three lines represent the simulations carried for three deletion constructs Epac1 (149-309), (161-309) and (173-309). The solid lines in panel d, are the secondary structures derived from the crystal structure of Epac2 CBD (PDB: 1O7F) with positive value as α -helix and negative value as β -sheet. All the measurements were carried out in a 0.1mM sample passed through a 100 kDa cut off filter. For R_1 the relaxation delays of 100 ($\times 2$), 200, 300, 400 ($\times 2$), 500, 600, 800, and 1000 ms ($\times 2$ indicates duplicate spectra) and for R_2 the relaxation delays of 8.48, 16.96, 25.44, 33.92, 42.4, 50.88, 59.36, 76.32, and 93.28 ms were used. For steady state NOE 10 sec recycle delay that included a 5 sec proton saturation period was used, while a recycle delay of 1.5 sec was used for the R_1 and R_2 experiments.

Figure 3) Reduced spectral densities for Epac1 (149-318) in its apo (orange) and cAMP-bound (black) states plotted against the residue number. The spectral densities were derived from the relaxation rates in Figure 2. a) Plot of $J(0)$ values that are sensitive to contributions from chemical exchange. Secondary structure elements are shown with positive and negative values as in figure 2. Residues with increase (decrease) in their $J(0)$ values are highlighted in red (blue). (b) Plot of $J(\omega_N)$ in which, $\omega_N = -\gamma_N \times B_0$ and $B_0 = 16.44$ T at 700 MHz. (c) Plot of $J(\omega_H + \omega_N)$ in which $\omega_H = \gamma_H \times B_0$. Residues for which $J(\omega_H + \omega_N)$ enhancement (reduction) upon cAMP-docking are highlighted in red (blue). In all panels, the red lines are calculations from the reduced spectral densities from the relaxation rates predicted based on the hydrodynamic simulation via HYDRONMR (6), as shown in Figure 2.

Figure 4) NMR relaxation dispersion (NMRD) measurements of Epac CBD with (black) and without (orange) cAMP. The relaxation rates were measured as $\Delta R_{2eff} = (1/T_{CP}) \ln(I_{472Hz}/I_{43Hz})$ where T_{CP} is the CPMG length of 93.3 ms. The I_{43} and I_{472} represent the peak intensity of resonances in the two CPMG field strength of 43 and 473 Hz.

Increase (decrease) in $\Delta R_{2\text{eff}}$, are highlighted with red (blue) columns and are representative of dynamic enhancements (quenching). Residues with $\Delta R_{2\text{eff}} < 2 \text{ S}^{-1}$ were shown with a downward triangle. Residues with $\Delta R_{2\text{eff}} + \text{error} > 2 \text{ S}^{-1}$ were shown with an elevated downward triangle at the maximum value ($\Delta R_{2\text{eff}} + \text{error}$). Positive and negative dashed lines on the top of the panel represent the secondary structures as described in figure 2. Three different ^{15}N carrier frequencies of 110, 119 and 127 ppm were used to eliminate the offset effects for the ^{15}N pulse.

Figure 5) 3D map of the dynamic patches in the apo Epac CBD. The dynamic data are mapped in to the 3D structure of Epac CBD (PBC:1O7F). $R_{1\rho} \pm \text{error}$ products above the 17.6 S^{-2} , which is the maximum value expected to be contributed via overall protein tumbling as predicted by hydrodynamic simulations are highlighted in red. All residues with $\text{NOE} \pm \text{error}$ values below 0.5 are highlighted in yellow. A277 and E308 possess both ps-ns and μs -ms dynamics and are highlighted in magenta. Regions important in Epac allostery are shown in box with dashed lines and the three ionic latch residues are shown with black circles.

Figure 6) 3D map of the effects of cAMP on the dynamic landscape of Epac CBD. The effects of cAMP on dynamics are mapped on the 3D structure of Epac CBD (PDB:1O7F). Increase (decrease) in μs -ms motions are highlighted in red (blue) and increase (decrease) in ps-ns motions are highlighted in magenta (cyan). I201 is enhanced in both μs -ms and ps-ns motions and is highlighted in orange. a) The zoom in view of the R279-G238 contact between PBC and $\beta 2$ - $\beta 3$ loop that are sandwiched between residues with quenched in dynamics. b) The zoom in view of the two ionic latch residues D172 and E197 in $\alpha 1$ and $\alpha 2$ with enhanced dynamics. c) Overlap of the apo Epac CBD (PDB:1O7F) in green and cAMP bound PKARI α CBD-A (PDB:1RGS) in cyan. The Hinge- $\alpha 4$ interface is shown with residues that show enhancement or quenching of dynamics.

Figure 7) A schematic representation of the model of EPAC activation. Epac exist in a dynamic equilibrium between the active (open) and inactive (close) states, which is affected by cAMP. The Epac1 CBD regions in which the overall dynamics is quenched by cAMP are colored red in the ligand free state and blue in the cAMP bound states. A reversed color code is used for the Epac1 CBD regions in which the overall dynamics is enhanced by cAMP.

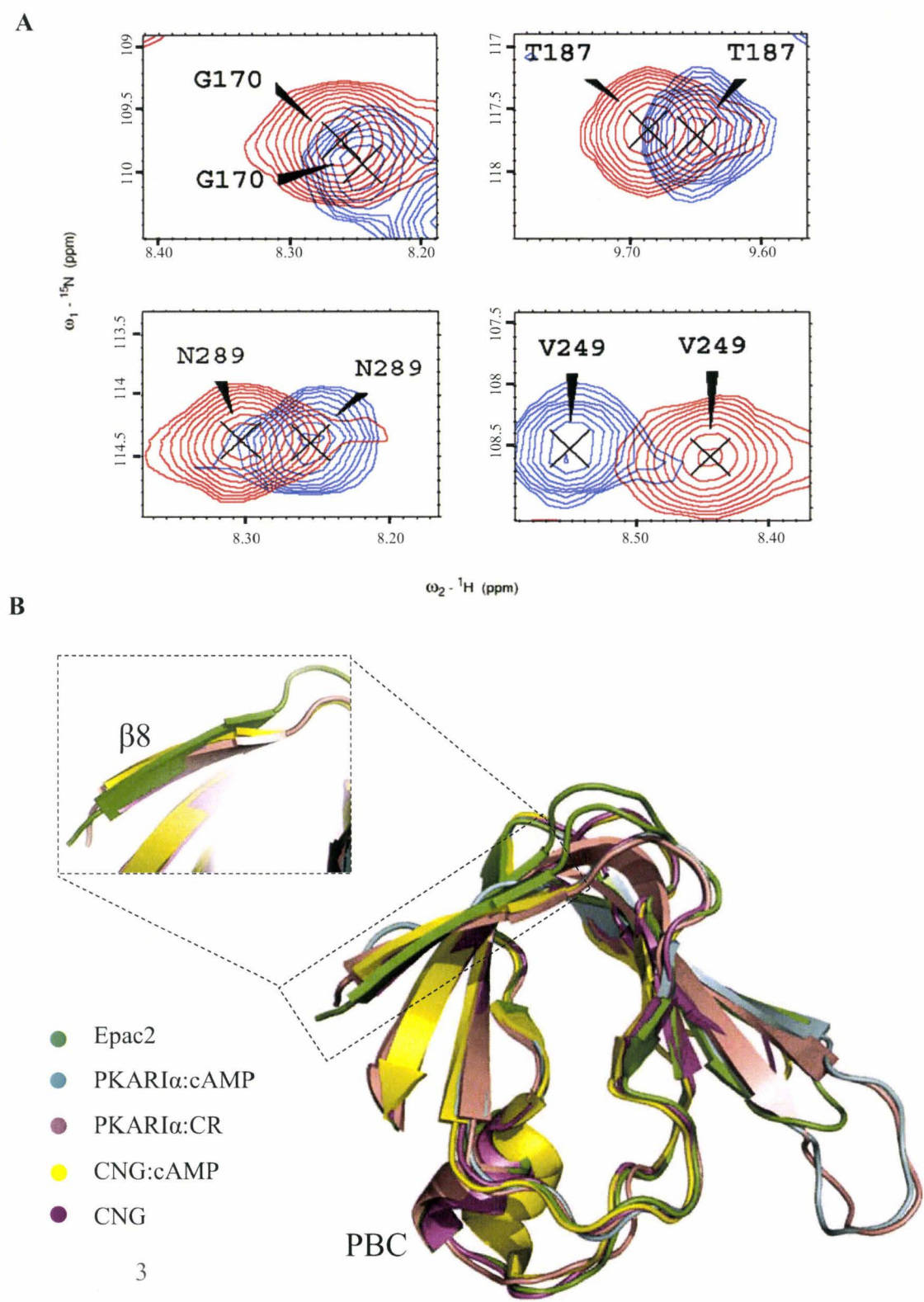


Figure 1

A

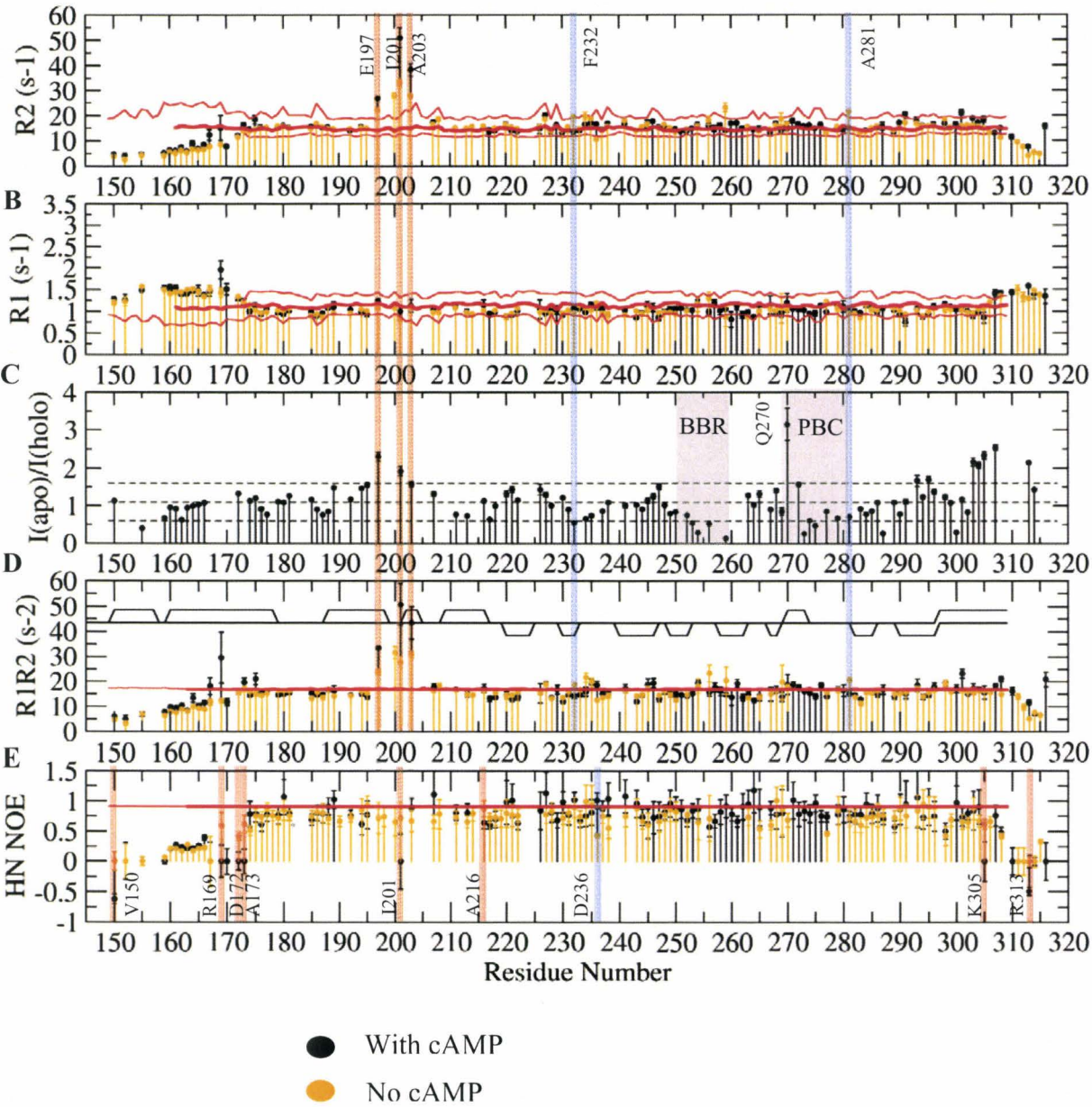


Figure 2

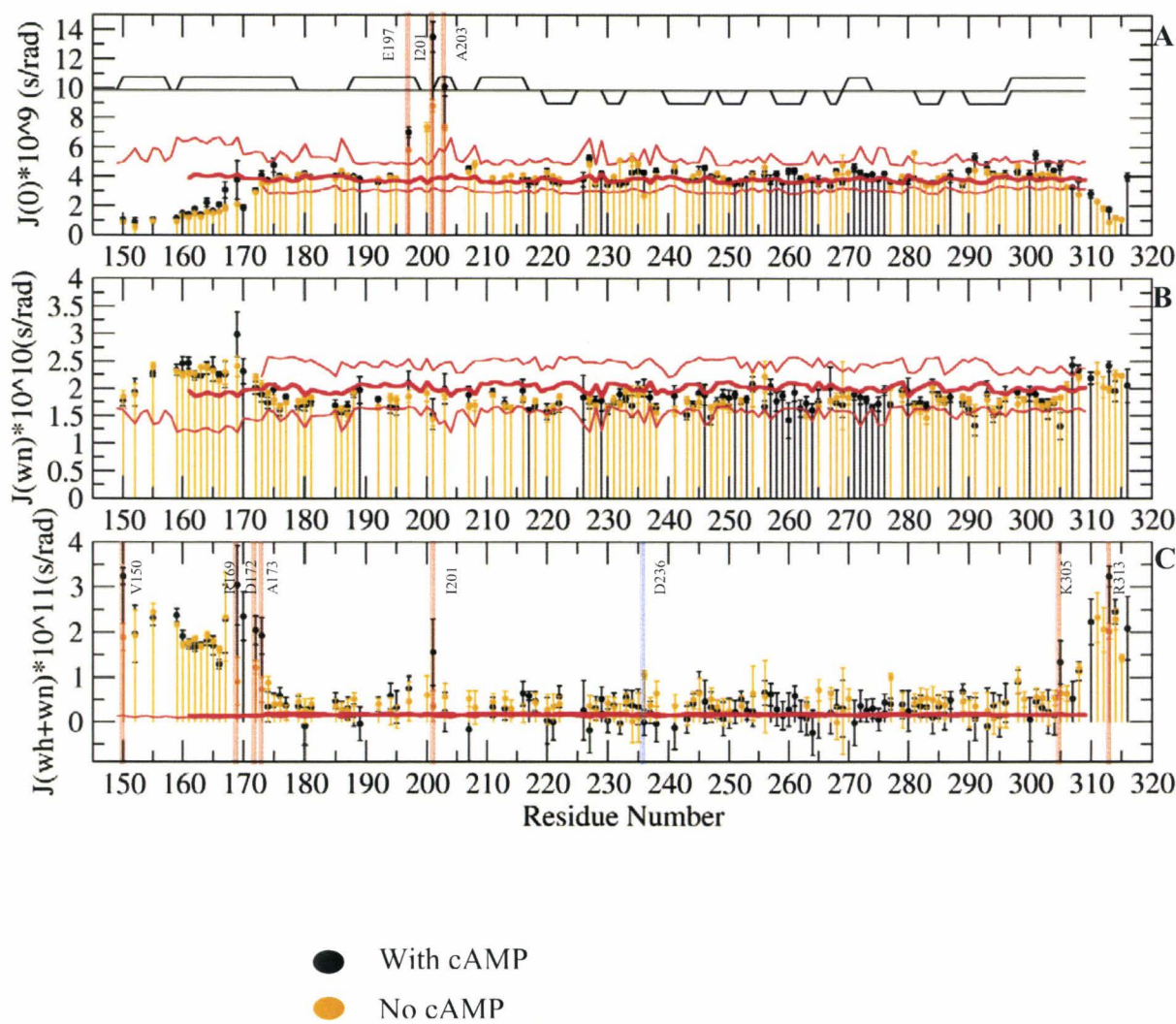


Figure 3

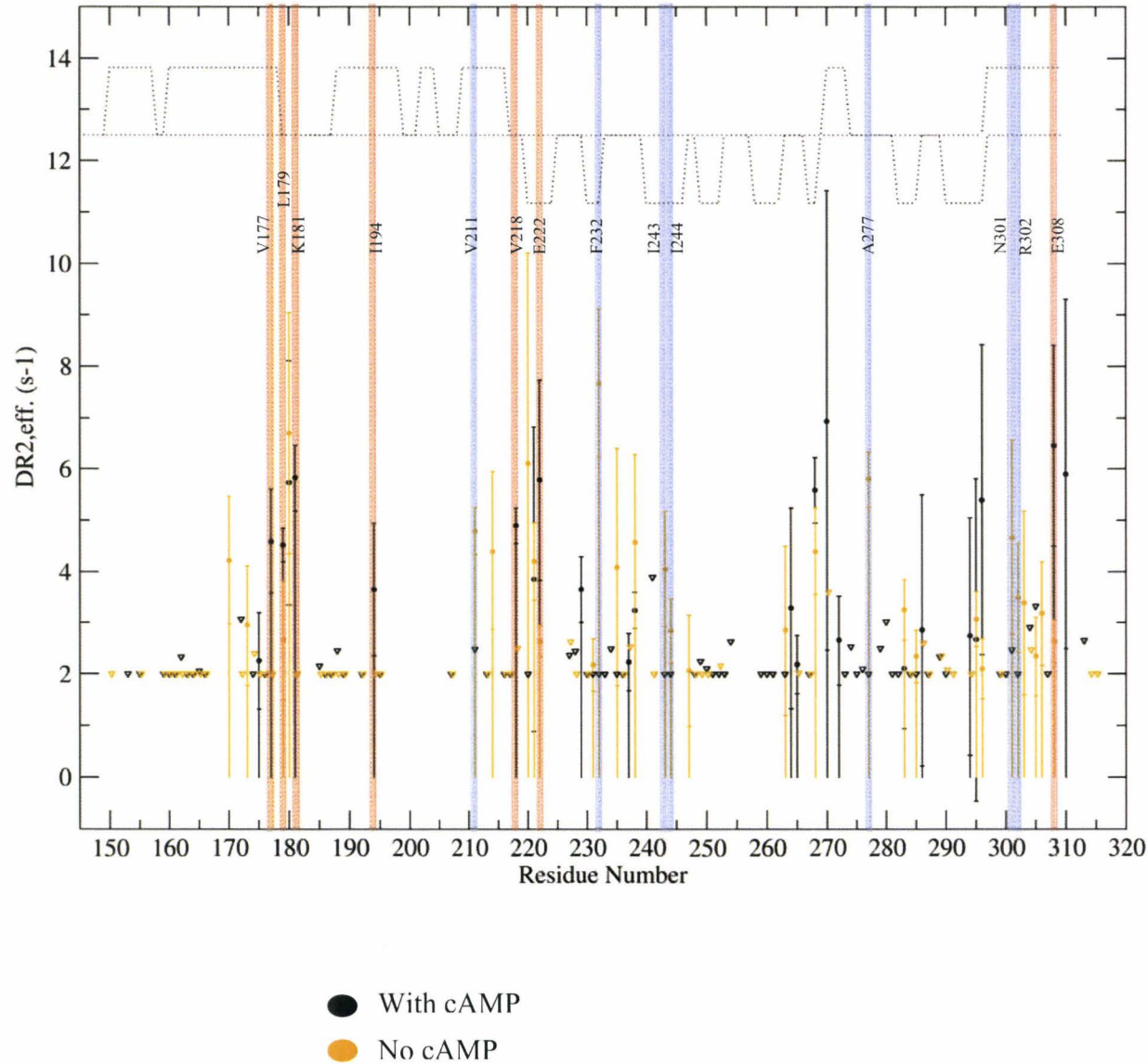


Figure 4

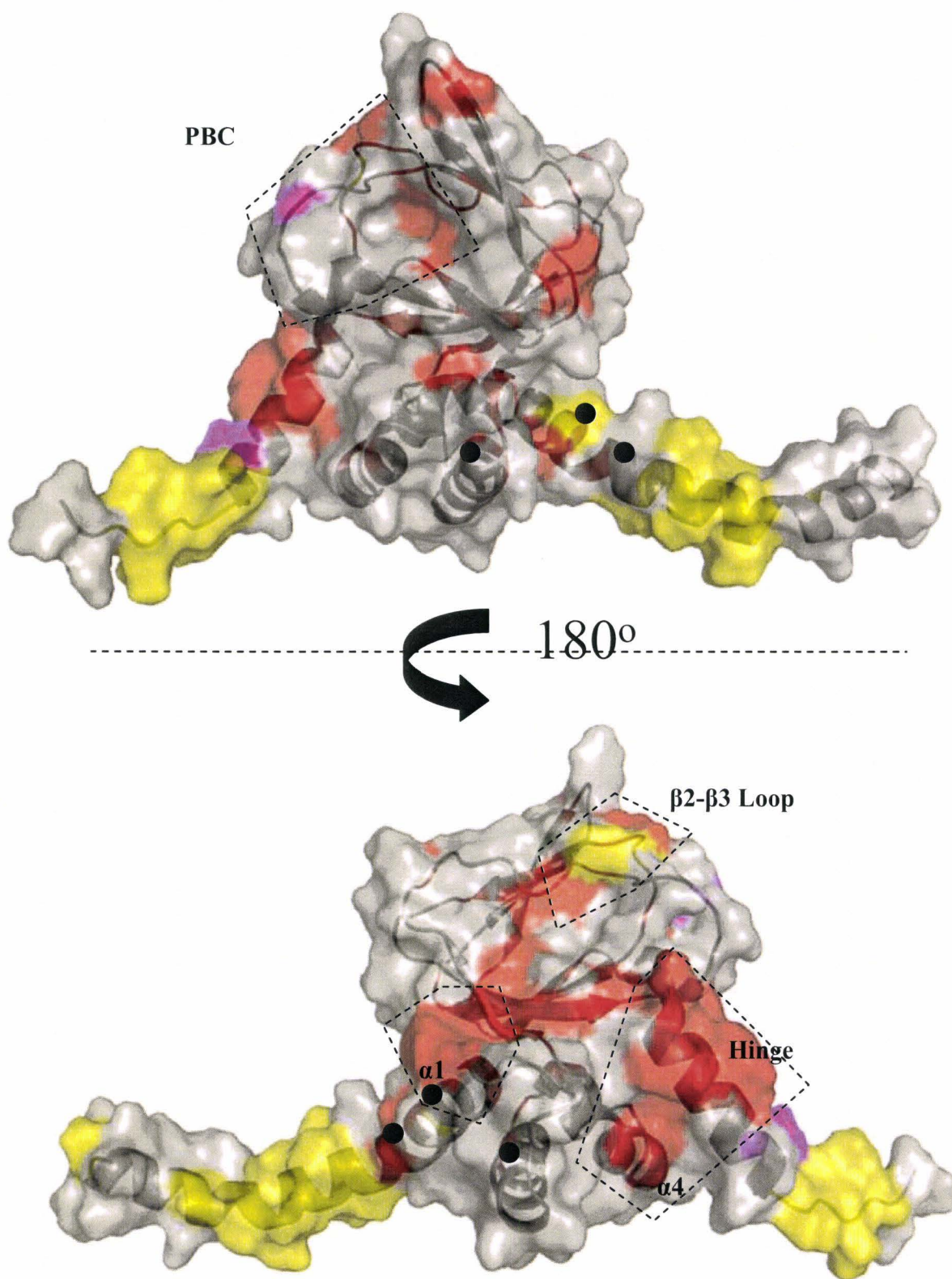


Figure 5

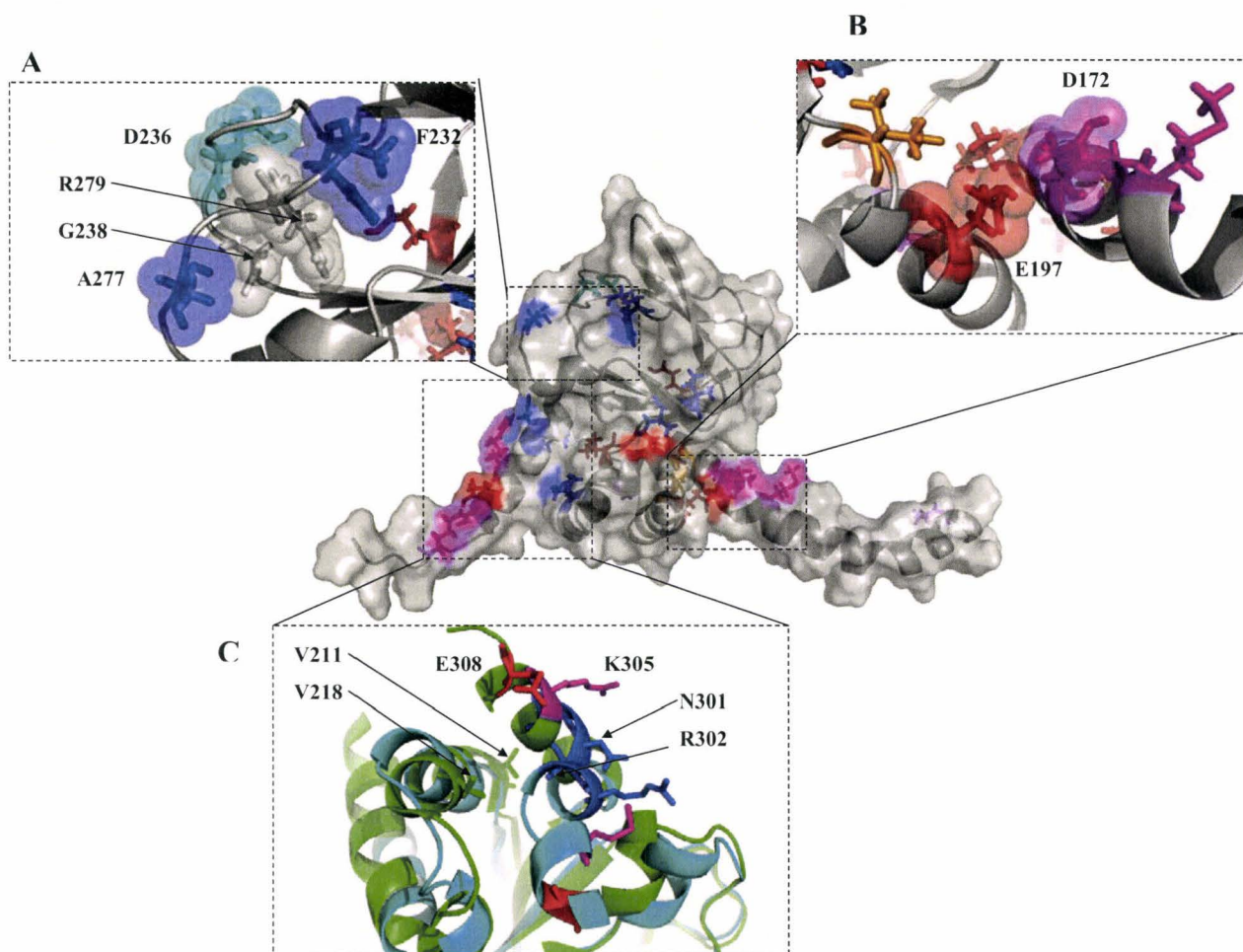


Figure 6

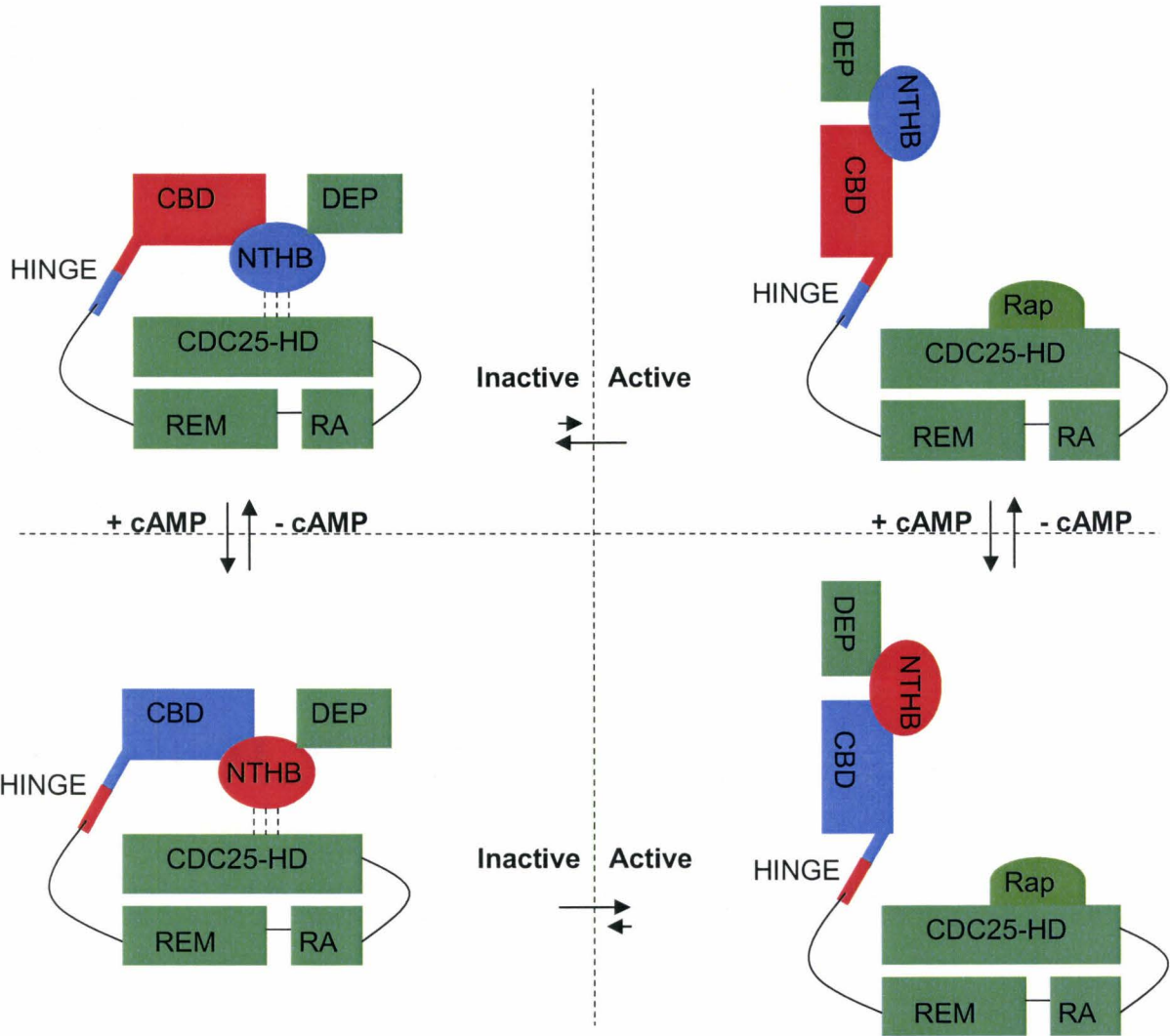


Figure 7

4.1 Allosteric Attenuator of Epac:

In this chapter we investigated the effects of the C-terminal segment of the Epac CBD that encompass the C-terminus of the Hinge and the Lid. Our initial understanding suggests that this segment attenuates the response of Epac toward cAMP by reducing the affinity of this CBD toward cAMP presumably by disruption of the hydrogen bonding network of the N-terminus of PBC that is known to interact with the phospho-ribose moiety of the nucleotide. In addition, the response to cAMP is further attenuated by thermodynamically disfavoring the conformation of the cAMP bound state presumably by increasing the enthalpy and decreasing the entropy. Third, the Lid attenuates by partial disruption of the communication network between the PBC and allosteric $\beta 2$ - $\beta 3$ loop in the liganded state. And fourth, by restricting the degree of freedom of the Hinge helix for inward rotation, toward the PBC, since we show evidence of intrinsic tendency of Hinge rotation toward PBC.

4.2 Introduction:

In chapter 2 we saw that upon ligand binding to the Epac CBD, the C-terminal Hinge-Lid region (CHLR) underwent a major structural re-arrangement. While the C-terminus of the Hinge helix was completely destabilized, the C-terminus of the Lid formed a transient helix with an averaged of 50% helix probability. In this chapter we constructed a CBD of Epac with the deletion of this loop-transient helix motif in order to assess its effects on the ligand recognition and allostery. We examine two constructs of Epac1 CBD, Epac 149-318 which contains the CHLR and Epac 149-305 which is devoid of this motif. We used multiple approaches in our investigation ranging from backbone chemical shift mapping, which provide information regarding the local backbone structure, hydrogen-deuterium exchange experiments, which assess surface accessibility and provide information on

exited states of the proteins, ^{15}N relaxation experiments, which provide information on multiple time scale dynamics, and mutational analysis on the CHLR and N-terminus of the PBC.

4.3 Results and Discussion:

4.3.1 CHLR dependent chemical shift variations: The ^1H , ^{15}N amide backbone compounded chemical shift were plotted for both Epac 149-318 and Epac 149-305 (Figure 1A). For a better representation of CHLR dependent chemical shift perturbations, direct comparison were made between the cAMP free and bound states of both constructs separately. In the absence of the CHLR the allosteric hot spots, Hinge and $\beta 2$ - $\beta 3$ loop, still display sensitivity toward cAMP binding, pointing to the intrinsic ability of these two sites in the relay of ligand signal to the downstream functional sites of the protein. Upon a brief examination of Figure 1A, two main differences can be observed between the two constructs. First, an additional region of CBD becomes sensitive to cAMP in the presence of the CHLR. This region spans $\alpha 3$ - $\alpha 4$ segment of the NTHB and according to the unliganded Epac2 structure it faces the CHLR (1). Second, the magnitude of change in the compounded chemical shift is on average higher in the longer construct compared to the shorter one. This suggests that the structural response of the CBD to cAMP is enhanced upon addition of the CHLR. Closer analysis of the chemical shift changes, reveal that the CBD backbone structure in the cAMP bound state is to a large extend insensitive to CHLR with the exception of $\alpha 5$ at the N-terminus of PBC (N-PBC), where the phospho-ribose moiety of cAMP is believed to be interacting, based on other known cAMP bound CBD structures (Figure 1A, forth panel). On the other hand, in the absence of the ligand a noticeable change in the CBD backbone conformation is introduced that encompasses all

sites sensitive to the cAMP with the exception of BBR (third panel). These observations assign the variations in cAMP mediated chemical shift seen between the two constructs primarily to the unliganded states. We sought to examine the ^1H and ^{15}N coordinates of these CHLR-sensitive resonances in the absence of the cAMP. The overlap spectra of a representative resonance show that in the unliganded, shorter construct the coordinates are closer to the bound coordinates, which suggests that the absence of CHLR results in a backbone conformation that is closer to the active/liganded form of the CBD (Figure 1B). This observation alone hint to the possibility that CHLR may confer a negative feedback in the process of Epac activation by shifting the Epac equilibrium state more toward the inactive conformation. In addition to this finding, the observed insensitivity of the BBR to the CHLR that is however 'seen' by all other cAMP responsive sites in the apo state (Figure 1A, third panel), might suggest that BBR is not connected to the allosteric circuit of the apo CBD.

The most pervasive change in chemical shift is seen for A280 at the C-terminus of the PBC. According to other known CBD structures, this residue is expected to form a hydrogen bond via its backbone amide with the axial oxygen of the phosphate group of the cAMP, which can account for its large chemical shift. But then what will account for its chemical shift difference between the two constructs in the absence of the ligand? Could it, in addition to ligand docking, be involved in allostery? In 2003, Bos and colleagues measured the affinity and activation of the mutant Epac1 A280T (2). This mutant is known to change the specificity of PKA from cAMP to cGMP, most probably via the formation of an extra hydrogen bond between the Thr and the 6-NH₂ group of the cGMP base (3). This mutation increased the affinity of Epac toward cGMP 4 folds. There was no change in

cAMP affinity, however, most interestingly; the $k_{\max}$ of this mutant was halved after activation with cAMP. This observation was suggested to show that A280 not only confers specificity in nucleotide recognition but it is also directly involved in relaying cAMP binding to Epac activation (2). A280 is C-terminal to R279 and might be able to communicate with the $\beta 2$ - $\beta 3$ loop, which can then see the R:C interface in NTHB.

4.3.2 CHLR modulations of the secondary structural probabilities: The effects of CHLR on the secondary structure of the β -subdomain and the NTHB portion of the α -subdomain are minor. However there are some interesting changes observed between the two constructs in this region (Figure 2); First, in the absence of the ligand the helical probability of the PBC helix are on average higher in the shorter constructs and resembles more of the cAMP bound conformation. This is in agreement with the chemical shift data and suggests that in the absence of CHLR the CBD adopts a conformation closer to the liganded/active state. Second, the BBR displays higher overall β -sheet probabilities in the shorter construct bound to cAMP compared to its longer construct counterpart, which might suggest that the mode of interaction between the cAMP base and BBR is modulated via CHLR. A close examination of secondary structural probabilities also reveals that the cAMP mediated destabilization of the $\alpha 3$ is independent of CHLR since the magnitude of this destabilization is unchanged in both constructs. One major difference between the CBDs is the Hinge helix, which is in extended conformation in the larger construct in the absence of cAMP and smaller by about five to six residues in the shorter one due to C-terminal truncation. An interesting note is that while Hinge and Lid are responsive to cAMP in terms of secondary structure, the Hinge in the Epac1 149-305 is to a large extent

insensitive. This could be explained by the fact that the part of the Hinge that is responsive is the C-terminus of this helix, which is deleted in the shorter constructs and by coincidence come in close proximity to the 'kink' point of the Hinge (I304-K305). Hence the bound and free length of the Hinge helix in Epac 149-305 is similar to that of the bound Epac 149-318. This might explain the compounded chemical shifts observed for $\alpha 3$ - $\alpha 4$ of the NTHB that directly faces the CHLR and is seen only in Epac 149-318. Hence, structural perturbations of the CHLR upon cAMP binding result in a magnetic environment similar to the one in its absence for residues in the NTHB that directly face the CHLR (Figure 1, forth panel). We might then infer that cAMP binding, to some extent, uncouples NTHB from CHLR.

4.3.3 CHLR dependent perturbations of the native state ensembles: The HD exchange rates of both CBD constructs were calculated as previously explained in Chapter 2. First, comparing the HD pattern in the presence of cAMP for both constructs reveals that overall, the presence of the CHLR does not affect the global unfolding energetics of the CBD since the buried residues in $\beta 3$ - $\beta 4$ and $\beta 7$ - $\beta 8$ with maximum protection from D₂O exchange have similar protection factor values (Figure 3A). However there are some local effects worth considering. First, the N-terminus of the Hinge shows major re-organization in the protection pattern between the two CBDs that point to a change in backbone hydrogen bounds. K305 becomes totally exposed to solvent and I303 and F300 gain protection upon the addition of CHLR. This suggests stronger backbone hydrogen bonds in the N-terminus of the Hinge helix conferred via CHLR. A very interesting observation is seen for residues at the N-PBC; while L271, A272, L273 and V274 all exhibit moderate to

low protection in the Epac 149-305, only A272 remains protected in Epac 149-318. Structural comparison with cAMP-bound CBD of LotiK1 channel (4) reveals that while there exist a tight backbone to backbone hydrogen bond network between these four residues, only A272 directly contacts the equatorial oxygen of the phosphate group of cAMP via its backbone (Figure 3B). In addition the side chain of R348 in the CBD of LotiK1, corresponding to E316 in Epac1, forms a salt bridge with the E298, corresponding to Q270 in Epac1, at the N-PBC. E316 is at the C-terminus of CHLR and may be able to bypass the truncation-mediated perturbations and still form a transient salt bridge. This can also explain the formation of a transient helix at the C-terminus of CHLR (Figure 2). Perturbations introduced at the side chain of Q270 can be transferred to adjacent residues and disrupt their backbone to backbone contacts. However, A272 contact with cAMP might be evolutionary conserved since it is necessary for the correct orientation of PBC for ligand docking. One would predict that this effect might have a consequence on ligand affinity and as a matter of fact we can observe a six fold increase in the K_D for cAMP upon addition of CHLR (Figure 9B). This goes in parallel with the hypothesis of the CHLR exerting a negative feedback in the process of Epac activation. Three selected regions also display increase in protection in Epac 149-305, which include G235 in β 2- β 3 loop, F221 in β 1, and A203 in α 3 helix. G235 forms a hydrogen bond with the carbonyl of R279 via its amide backbone, which is a critical contact between PBC and the β 2- β 3 loop. The other critical backbone to backbone contact between R279 amide and D236 carbonyl is unperturbed. In the cAMP bound conformation of homologues CBDs, the N- and C-terminus of PBC are coupled via the phospho-ribose moiety of cAMP, hence destabilization at the N-terminus of the PBC is transmitted to its C-terminus in contact with

β 2- β 3 loop. This might suggest that the CHLR not only decreases the affinity toward cAMP but also attenuates the allosteric communication network between PBC and β 2- β 3 loop. F221 and A203 face the C-terminus of β 8 and the Hinge helix respectively and their perturbations can be directly mediated by the CHLR.

In the cAMP bound conformation with the absence of the CHLR, there are more protections for residues in the α - and β -subdomains, with the exception of the Hinge. All protections gained in Epac 149-305 are considered low to moderate since their PF values range from 2.5 to 5.5. As stated previously in Chapter 2, residues with maximum PF values of 8-9 probe exchange competent states via global unfolding fluctuations and the ones with lower PF values can probe those states through the faster mechanism of local unfolding fluctuations, which are usually in slow ms exchange regime (5). This suggests that although absence of the CHLR results in the formation of the aforementioned protections, probably via hydrogen bonds, those regions possess slow ms motions to exchange competent states.

In the absence of cAMP, D296 at the beginning of the Hinge is protected in the absence of CHLR and become exposed in the longer construct (Figure 4). D296 have on average 7.5 Å distance from the carbonyls of F300, D299 and Q298 in the cAMP free structure of Epac, which will hamper the formation of any backbone to backbone contact. However one can imagine that if the hypothesis of Epac activation is correct and indeed the Hinge comes in close proximity to the PBC upon ligand binding, the aforementioned distances would decrease. D296 is protected in the liganded/active conformation in both constructs; hence the movement of the Hinge might provide the protection of D296 amide backbone from solvent exchange. This suggests that in the absence of CHLR the Hinge

might adopt a conformation closer to that of the active one, which is also supported by our compounded chemical shift data and the presence of a minor population of Hinge resonances in the unliganded Epac 149-305 that sample the liganded state of this construct (Figure 9C). Four residues within the β -subdomain also possess higher protection in the shorter construct. These include K246-S248 in the $\beta 3$ - $\beta 4$ loop and L262-G265 in the $\beta 5$ - $\beta 6$ loop. These residues are adjacent to each other in the Apo structure with an average distance of 7 Å (Figure 4B). It is interesting that all four become protected in the presence of cAMP in both constructs. cAMP confers a more compact structure in the β -subdomain as judged by higher overall PF values. It is possible that these four residues, in the core of the domain, become more protected in the presence of CHLR-deletion-induced cAMP-like conformation in the unliganded state.

4.3.4 CHLR-dependent dynamic modulation in Epac CBD: We have compared the multi-time scale dynamics of Epac 149-305 and Epac 149-318 with and without cAMP in order to explore in more detail the affects of the CHLR. Overall the R1 and R2 values are higher and lower respectively for the smaller CBD due to the faster overall molecular tumbling (6). Therefore, we only used $R1 \times R2$, $\{^1\text{H}\}-\{^{15}\text{N}\}$ steady state NOE (HN-NOE) and NMRD for comparison between the two constructs which are not significantly affected by differential molecular tumbling.

Comparing both constructs in the absence of cAMP (Figure 5&6), Epac 149-305 displays enhanced dynamics in μs -ms and ps-ns time scales for both α - and β -subdomains relative to the longer construct. The μs to slow ms dynamics are mapped primarily to the PBC, $\beta 2$ - $\beta 3$ loop, C-terminus of $\beta 1$ and $\alpha 3$ - $\alpha 4$ regions and the ps-ns motions are mostly within the $\alpha 1$ and C-terminus of Hinge helix. PBC is in direct contact with the Hinge

through the hydrophobic bridge of F300-L273. The $\beta 2$ - $\beta 3$ loop can be indirectly coupled to the Hinge via R279 in the PBC and directly via K297 at the N-terminus of the Hinge (see the contact network in Chapter 2). The $\alpha 3$ - $\alpha 4$ region forms the interface between NTHB and the Hinge helix and the C-terminus of $\beta 1$ can be modulated via the N-terminus of $\beta 8$ which is in contact with $\alpha 2$ - $\alpha 3$ segment of NTHB. The Hinge in the absence of CHLR is affected by both μ s-ms and ps-ns motions, also F300, I303 and K305 display double conformation behavior in the HSQC spectra implying slow ms dynamics (Figure 6C). Also I304 and K305 have higher $\Delta R_{2,\text{eff}}$ compared to those in the presence CHLR (Figure 6A). It is then possible to argue that the overall multi-time scale enhancement in dynamics observed in the Apo Epac 149-305 in both subdomains may have been initiated via the Hinge helix. The pattern of dynamics also suggests that slower motions tend to focus within the β -subdomain and selected regions of the α -subdomain such as $\alpha 3$ - $\alpha 4$ segment and Hinge helix. According to chemical shift and hydrogen-deuterium exchange data, Apo Epac 149-305 is expected to be closer to the cAMP bound conformation when compared to the longer Apo construct. In Chapter 3 we showed that the active conformation is characterized by overall dynamic enhancement and quenching of the α - and β -subdomain respectively. Hence, if the absence of CHLR shifts the equilibrium of inactive $\rightarrow$ active conformation forward, we would expect a dynamic profile more similar to the cAMP bound Epac 149-318 compared to its Apo counterpart. This is true in the case of the α -subdomain where we observe more μ s-ms and ps-ns dynamics located in the proximity of ionic latch residues, such as L165, T176 and I201. However, the β -subdomain does not show signs of dynamic quenching and as a matter of fact it displays higher $R_1 \times R_2$ and $\Delta R_{2,\text{eff}}$ values, indicating the presence of μ s to slow ms motions in the absence of CHLR.

The explanation could be that the absence of CHLR does not results in a major shift in the equilibrium state of the CBD and is less compared to the one conferred via cAMP, as suggested via the chemical shift data (Figure 1A, compare first and third panels). This could result in the formation of transient population of structures that probe either the active or inactive states. These transitions can happen in slow time scales that provide the ability of hydrogen bond formation. As the HD exchange data suggest there are several additional low to moderate protection observed in the Apo Epac 149-305 which could be explained by this hypothesis.

Comparing the cAMP bound dynamics in the presence or absence of CHLR, the α -subdomain tends to be slightly more dynamic in ps-ns and μ s-ms in NTHB in the shorter construct (Figure 7). However, the increase in entropy in NTHB do not tend to focus on ionic latch residues, hence in the bound conformation the dynamic level of Q168, D172 and E197 is enhanced via cAMP to approximately the same extend regardless of the presence or absence of CHLR. This is in accordance to our chemical shift and HD data which suggest that the structure and solvent accessibility of cAMP bound Epac 149-305 and Epac 149-318 are similar within the NTHB in the presence of cAMP. In Chapter 3 we showed that the primary mean of the cAMP-mediated control of the ionic latch is through dynamics. It is then apparent that the N-terminus of Hinge helix and β 2- β 3 loop can form sufficient contacts with PBC and NTHB to provide an adequate path for the relay of cAMP signal to the ionic latch both in the shorter and longer constructs. The observation that the overall dynamic of NTHB is slightly attenuated in the presence of the CHLR is interesting in itself. The compounded chemical shift data suggested the presence of a negative feedback conferred via CHLR on the Apo structure that keeps the unliganded conformation

further away from the liganded one. It is then possible that, even in the presence of cAMP, CHLR tends to shift the equilibrium activation of Epac to the inactive form. The Hinge helix exhibits mix patterns of dynamic enhancement in ps-ns, I304 and K305, and quenching in μ s-ms, N301, in the absence of CHLR. Within the β -subdomain there are selected regions that show enhanced dynamics in μ s-ms and ps-ns motions in the active form. N275, D276 within PBC and K256, L258 within BBR display higher μ s-ms motions. These regions are in direct contact with phospho-ribose moiety and base of the cAMP respectively and their perturbations can be the results of direct/indirect effect of CHLR on cAMP. D236 in the β 2- β 3 loop, which is in contact with R279 of PBC, possess slightly higher ps-ns dynamic in the absence of the CHLR. However this enhanced dynamic is not significant since D236 retains the same level of protection from D₂O in the absence and presence of CHLR.

HD data, in the presence of cAMP, suggested the presence of slow ms fluctuations within the β -subdomain in the absence of CHLR. It is interesting that the dynamic data also point to the same pattern. Why then the dynamic of this subdomain not quenched to the same extend as the CHLR containing construct in the cAMP bound state? The answer is not precisely known at the moment but for speculation; if the hypothesis regarding the negative feedback mechanism of CHLR is true, then one might argue that in the Epac 149-305 the inverse dynamic relationship between the two subdomains are attenuated to some degree, hence increase in dynamic of α -subdomain do not come at the cost of decreased entropy of the β -subdomain. CHLR disrupts several hydrogen bonds at selected loci and decrease the dynamics through out the β -subdomain, so it increases the enthalpy ($\Delta H > 0$)

and decreases the entropy ($\Delta S < 0$), hence increasing the free energy of the cAMP bound conformation ($\Delta G = \Delta H - T\Delta S > 0$), which is not thermodynamically favored.

4.3.5 Evidence of the interaction of Lid with N-PBC: A detailed comparison of Epac in the presence and absence of CHLR has shed light on the possibility of direct interaction between Lid and the N-PBC. There are several evidences pointing to this potential interaction. First, the intensity of the Q270 amide backbone is sensitive to perturbations in the Lid region (Figure 8A). Upon deletion of CHLR, the intensity is significantly enhanced, which suggests the quenching of slow ms motions. Second, the N-PBC hydrogen bonding network is disrupted in the presence of CHLR. Third, the dynamic of the C-terminal E316 in the Lid contain both fast ps-ns and slow μ s-ms dynamics (Figure 7). The slower motions suggest that C-terminal Lid might have an interacting partner in the cAMP bound conformation. Fourth, upon ligand binding, the Hinge-Lid region adopts a helix-loop-transient helix motif structure as judged by backbone chemical shifts (Figure 2). This again hints to the possibility of the existence of an interacting partner for the C-terminal Lid. According to other known cAMP bound CBD structures (7), E316 in Epac1, might come in close proximity to Q270. Since the side chains of these two residues contain apposite charges, there exist a potential for salt bridge formation. Fifth, in the presence of the CHLR, the affinity of the CBD toward cAMP is reduced 6 times (Figure 9B). And finally sixth, upon mutation of Q270 to E, in addition to the perturbations of flanking residues within the sequence, selected residues in the beginning of CHLR were also perturbed (Figure 9C). This is the region where Hinge helix is presumably kinked upon cAMP binding, in order for the Lid to bend toward the N-PBC.

In 2003, Q270 was proposed to disfavor the active conformation of Epac1 CBD (2). The reason was the observation that upon Q270A mutation, while the K_D remained unaffected, the K_{max} of Epac1 activation was increased by 70%. It is then probable that the negative feedback exerted by CHLR is to a certain extent mediated via interaction with Q270. Here we can bring in to play the Epac super activator, 8-pCPT-2'-O-Me-cAMP. This cAMP analog is known to shift the equilibrium state of Epac to the active side more than that of cAMP since the K_{max} is tripled and the AC_{50} is reduced 25 times compared cAMP (2). It is proposed that the 2'-O-Me of this analog communicates with Lid to bring about the super activation. This is supported by the observation that cAMP and 8-pCPT-cAMP are differentially affected by Lid mutations compared with the 8-pCPT-2'-O-Me-cAMP. While R313A did not affect the K_{max} of cAMP and 8-pCPT-cAMP, it reduced the K_{max} of the super activator by 50% (2). T311A elevated the K_{max} of cAMP and 8-pCPT-cAMP by 50% but did not affect the K_{max} of the super activator (2). Hence if the super activator recruits the Lid in the activation process, mutations that might eliminate potential interacting partners of the Lid, may hamper the super activation. As a matter of fact Q270A results in 40% reduction in K_{max} of 8-pCPT-2'-O-Me-cAMP, an opposite effect for cAMP. And as mentioned above T311, which has been recently proposed by molecular simulation from Xiaodong Cheng group to form an ionic bridge to Q270 (8), upon mutation to A doubled the K_{max} of cAMP but did not affect the K_{max} of the super activator (2). These data strengthen our hypothesis of the existence of Lid-Q270 interaction that might exert a negative impact in the process of Epac activation.

Interaction of Lid with the N-PBC might have an evolutionary explanation. The CBD of Epac is assumed to have been evolved from that of the PKA, where the affinity of the

isolated CBD toward cAMP is in low nM range (7). However the isolated CBD of Epac has affinity in low μ M range. How this increase in K_D for cAMP occurred is not fully understood. It has been proposed that several adaptations have contributed to this decrease in affinity (9). First, the apparent lack of the necessity of hydrogen bond between Q270 and the 2'-OH group of cAMP. Second, the apparent lack of proper stacking interactions between Lid and the base of the cAMP, as suggested by molecular simulations (9). And third, the difference in space, hydrophobicity and side chain dynamics between the PBC of Epac and PKA. The observation that addition of CHLR decreases the affinity toward cAMP could be within the same evolutionary trend of diminishing cAMP affinity.

We also performed some preliminary analysis of Epac 149-310 in which the Hinge helix is complete and Lid is deleted, as inferred from the Epac 149-305 and Epac 149-318 PECAN analysis. This protein behaved more similar to Epac 149-305, which suggests that the effects of CHLR described above might also be mimicked in the absence of the Lid alone (Figure 1B). However more data on Epac 149-310 construct is required before this can be ascertained. For example it is possible that the C-terminus of Hinge is still destabilized in the intermediate construct in the absence of cAMP.

4.4 Conclusion:

In this chapter we showed that CHLR impacts negatively the ligand association and the allostery in Epac proteins. The negative feedback of CHLR can be divided in two categories; first, this motif tends to influence the unliganded conformation of CBD to a more distant structure from the cAMP bound conformation. A look at the crystal structure of Epac2 in the absence of cAMP, reveals that the switch board region C-terminal to Hinge, is a hydrophobic glue that keeps the Hinge away from the CBD (Figure 10A). It is

probable that the Hinge helix contains an intrinsic tendency to partially swing to the core of the CBD and hence the switch board is counteracting this tendency. Mutations that abolished the hydrophobic core of the switch board, VLVLE → AAAAA, resulted in activation of Epac in the absence of cAMP (Figure 10B) and recent mass spectroscopic based hydrogen-deuterium exchange data on Epac1 have shown that switch board undergoes a major re-organization upon ligand docking (1, 8). It is possible that in Epac 149-318 construct, the additional residues in Lid necessitate more energy for inward rotation of the Hinge, hence to some extent counteracting the intrinsic tendency of the Hinge helix to swing toward the PBC (Figure 9A). Second, in the liganded structure, this motif most notably influences the Epac activation attenuator, Q270. Hence it is possible that at least a portion of the negative feedback is conferred via interaction of Lid with Q270. The effect of CHLR on the N-PBC can influence the cAMP docking as indicated by 6 fold reduction in ligand affinity introduced by CHLR. The CHLR might also thermodynamically disfavor the cAMP bound conformation by increasing the state's enthalpy and decreasing its entropy.

4.5 Acknowledgements:

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4.7 Figure Captions:

Figure 1) Chemical shift mapping of Epac CBD. A) The compounded chemical shift of the amide backbone of Epac CBD constructs is plotted against the residue number. The first two panel from the top are compounded chemical shifts of Epac 149-318 and Epac 149-305 in response to cAMP, respectively. The last two panels are compounded chemical shift map between the two constructs in the absence (third panel) and presence (fourth panel) of cAMP. Regions responding to cAMP and CHLR are highlighted in red and blue columns respectively. B) left panel; a representative resonance of a amide backbone (G238) is shown in Epac 149-318 with (blue) and without (red) cAMP. The corresponding resonance in Epac 149-305 in the absence of cAMP is shown in green. Right panel; similar to left spectrum with the addition of G238 resonance in Epac 149-310 construct in the absence of cAMP shown in yellow.

Figure 2) Secondary structural analysis of EPAC CBD with and without cAMP. The probabilities of the secondary structures are plotted against the residue number. Positive value is indicative of α -helixes and negative value that of β -sheets. In the upper panel the plot is shown in the absence cAMP for Epac 149-318 in black and Epac 149-305 in red. The second panel is the same as the upper but in the presence of cAMP. The lower two panels are the differences in secondary structure upon cAMP binding. The second panel from the bottom is the variations seen in α -helixes and the bottom panel for the β -sheets. Positive values in the last two panels represent enhancement in that secondary structure upon ligand binding and the negative value means the reverse. The solid lines on the upper panel are secondary structures derived from the crystal structure of Epac2 with positive for α -helixes and negative for β -sheets.

Figure 3) The effect of CHLR on HD exchange in the presence of cAMP and the hydrogen binding network of N- and C-terminus of PBC with cAMP. A) The HD exchange profile of Epac 149-318 (in black) and Epac 149-305 in orange is shown for cAMP bound states. The protections factors are plotted on the y-axis in logarithmic scale against the residue number. The residues affected by the CHLR in terms of their HD exchange are indicated in black (gain of protection in the presence of CHLR) and blue (loss of protection in the presence of CHLR). Circles are indication of fast exchange, squares are indication of overlaps and upper and lower arrows are indication of upper and lower limits respectively. B) This figure was made using the PYMOL software (10) with the LotiK1 channel CBD (^{PDB: 1VP6}) (4). The critical hydrogen bounds are shown with dashed lines and are discussed in the text. For each residue in LotiK1 channel the corresponding residue in Epac is indicated with a star. The sequence was aligned using the CLUSTALW sequence aligning software (11). The interaction between the R348 in the LID with the N-PBC (E298) is also shown. Green, red, blue and orange represent carbon, oxygen, nitrogen and phosphate respectively.

Figure 4) The effect of CHLR on HD exchange in the absence of cAMP and 3D mapping of CHLR effects with and without cAMP. A) As in figure 3A but in the absence of cAMP. B) The effects of CHLR are mapped on the 3D structure of Epac2

CBD-B crystal structure^(PDB: 2BYV) (1). The left panel is the effects of CHLR in the presence of cAMP and the right in the absence of cAMP. The color coding is the same as in figure 3A. Residues that are conserved between Epac1 and Epac2 are numbered according to Epac1 sequence. Residues that are not conserved between the two Epac isoforms are indicated by a slash in which the residue in Epac 2 is indicated with a subscript m.

Figure 5) Comparison of relaxation profiles between Epac 149-305 and Epac 149-318 in the absence of cAMP. A) The R2, R1, R1×R2 and steady state NOEs are plotted for cAMP free state in the absence (orange) and presence (green) of CHLR against the residue number. The black circles are relaxation rates in the presence of cAMP and CHLR. Residues selected via blue columns are affected by dynamic quenching in ps-ns time scale imposed in the absence of CHLR and those in red column are affected by enhanced dynamics in μs-ms time scale in the absence of CHLR. Residues in pink columns are enhanced in ps-ns motions in the absence of CHLR. The red lines are values computed based on hydrodynamic modeling of the overall tumbling of the protein^(PDB: 1O7F) via the HYDRONMR software in order to identify possible contributions from diffusional anisotropy and the overall tumbling of the protein to the relaxation rates (12). B) 3D map of the dynamics crystal structure of Epac2 CBD-B^(PDB: 2BYV) (1). Residues that are conserved between Epac1 and Epac2 are numbered according to Epac1 sequence. Residues that are not conserved between the two Epac isoforms are indicated by a slash in which the residue in Epac 2 is indicated with a subscript m.

Figure 6) Comparison of slow ms dynamics between Epac 149-305 and Epac 149-318 in the absence of cAMP. A) The color coding for the relaxation rates is as figure 5. Red columns show enhanced in slow ms motions in the absence of CHLR and blue columns quenching of such motions. B) 3D map of the dynamics in slow ms time scale with the same color coding as in A on the crystal structure of Epac2 CBD-B^(PDB: 2BYV) (1). Residues that are conserved between Epac1 and Epac2 are numbered according to Epac1 sequence. Residues that are not conserved between the two Epac isoforms are indicated by a slash in which the residue in Epac 2 is indicated with a subscript m.

Figure 7) Comparison of relaxation profiles between Epac 149-305 and Epac 149-318 in the presence of cAMP. A) As in figure 5. Red columns indicated more μs-ms, pink indicates more ps-ns and green quenching of μs-ms motions in the absence of CHLR. B) 3D map of dynamics on the crystal structure of Epac2 CBD-B^(PDB: 2BYV) (1). Residues that are conserved between Epac1 and Epac2 are numbered according to Epac1 sequence. Residues that are not conserved between the two Epac isoforms are indicated by a slash in which the residue in Epac 2 is indicated with a subscript m.

Figure 8) The mutational evidence for the interaction between LID and the PBC. A) The intensity of Q270 was measured in the presence and absence of cAMP for the constructs with and without the Lid and several different Lid mutants. The ratio of I_{Holo}/I_{Apo} was then normalized relative to the resonance of the S248 which is relatively insensitive to that of cAMP. Spectral noise was used in error estimation. B Schematic presentation of the

proposed Lid interaction with the N-PBC as inferred from both mutational and simulation and sequence alignment analysis (1).

Figure 9) The effects of the CHLR on the equilibrium activation of Epac CBD. A) A schematic presentation of the effects of the CHLR addition on the Epac CBD with various C-terminal deletions. The gray triangle on the Epac 318 signifies the random motions of a flexible peptide that can restricts the degrees of freedom of the Hinge helix. B) The cAMP affinity measurements on Epac 149-318 and Epac 149-305 with saturation transfer difference (STD) amplification factor. All STD measurements were carried out with a solution of 15 μ M protein, either Epac1 (149-305) or Epac1 (149-318) in 20 mM phosphate buffer, pH 7.6, 50 mM NaCl, 99.9 % D₂O and at 25 °C. The 1D-STD spectra were acquired at total cAMP concentrations of 25, 50, 75, 100, 150, 200 and 300 μ M (12). Separate reference 1D-ST (STR) experiments were also acquired. The STD amplification factor (STD_{af}) was calculated as the product of the STD/STR ratio, measured for the well resolved cAMP ribose H1' at 6.2 ppm, and of the ratio of the total cAMP and protein concentrations (12). The STD_{af} values were then normalized relative the STD_{af} plateau value reached at high cAMP concentrations such as [cAMP_{Tot.}] $\geq$ 150 μ M. The normalized STD_{af} values were then analyzed with the binding isotherm equation: Normalized STD_{af} = $1 - (1 / (1 + ([cAMP]/K_D)))$, where [cAMP] is the concentration of free cAMP (12). The solid line on Epac 149-305 represents the K_D of 3 μ M, while the dashed lines correspond to K_D values of 1 and 5 μ M. C) left: the presence of minor population in the unliganded Epac 149-305 (red) that probe the liganded state of this construct (blue). Right: the HSQC spectra of Epac 149-318 Q270 mutant, with the effects of the mutation in circle.

Figure 10) A possible mode of Epac activation. A) The hydrophobic glue of the switchboard (SB) that can keep the Hinge away from the core of the CBD is shown with residues that surround each of the residues in the conserved VLVLE sequence. Each of the VLVLE residues is given a number and the residues that surround it are given in the parenthesis. The PDB: 2BYV was used to generate this figure (1). B) The mode of Epac activation in Epac VLVLE $\rightarrow$ AAAAAA mutants. This mutant could disrupt the hydrophobic core of the switch board, giving the Hinge more degree of freedom to move toward the core of the domain. The VLVLE sequence within the SB is shown by dark blue and the loss of the hydrophobic core of SB by mutation is shown via outward arrows.

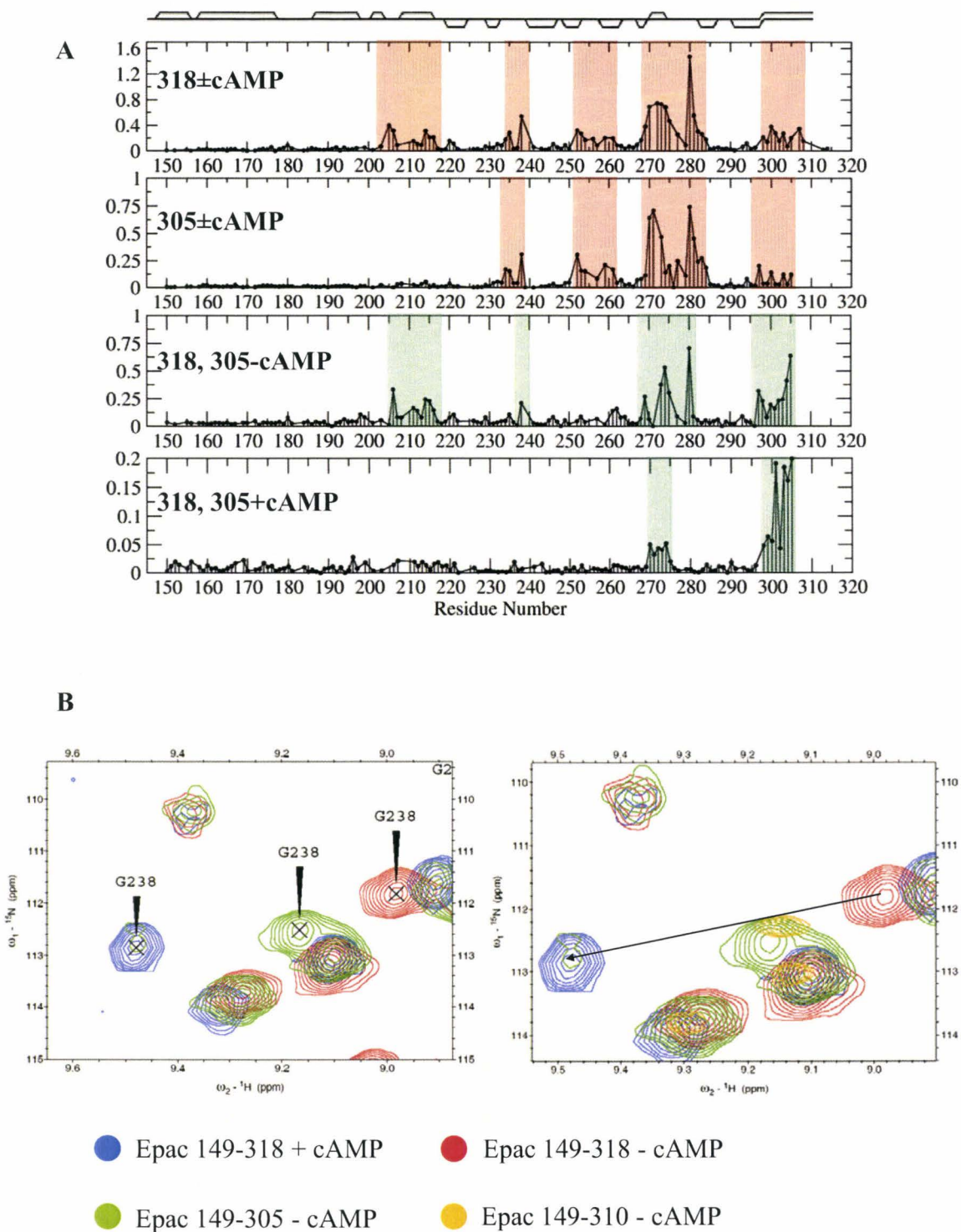


Figure 1

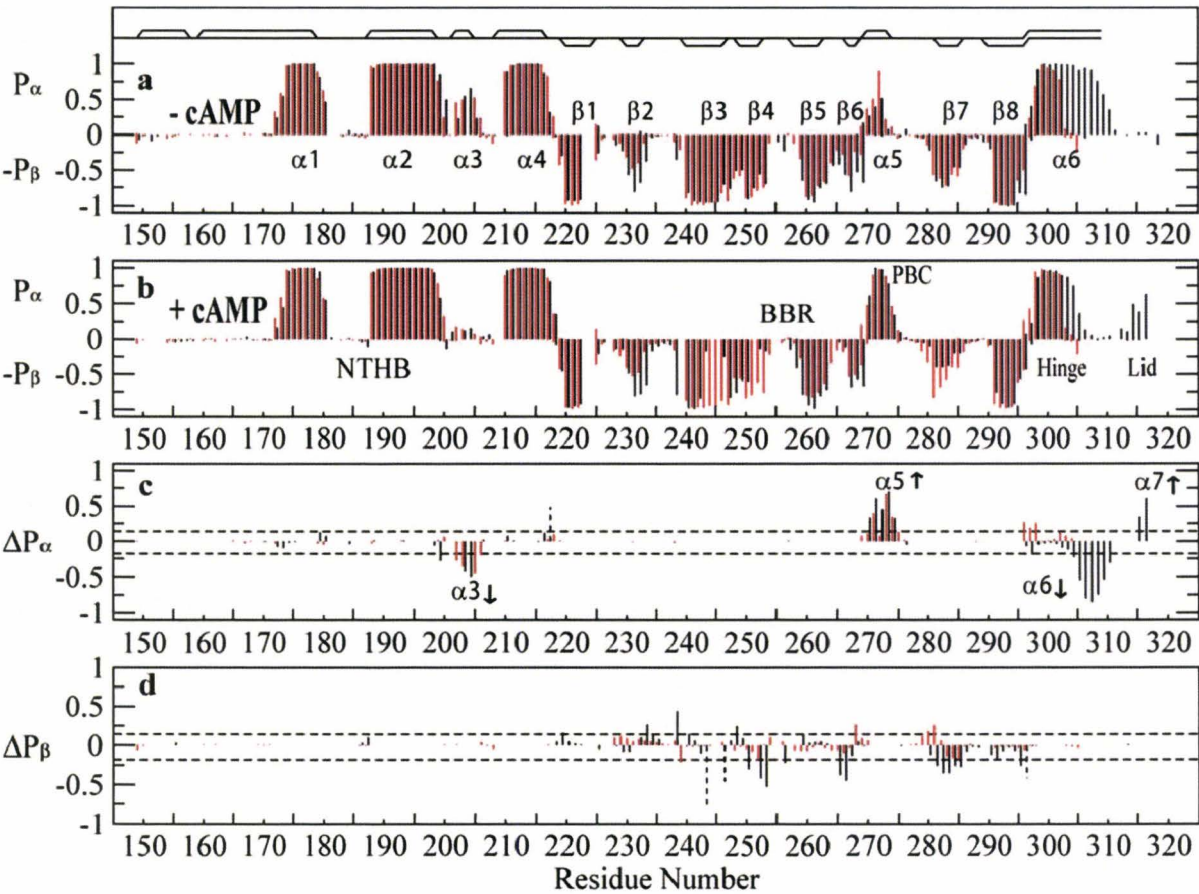


Figure 2

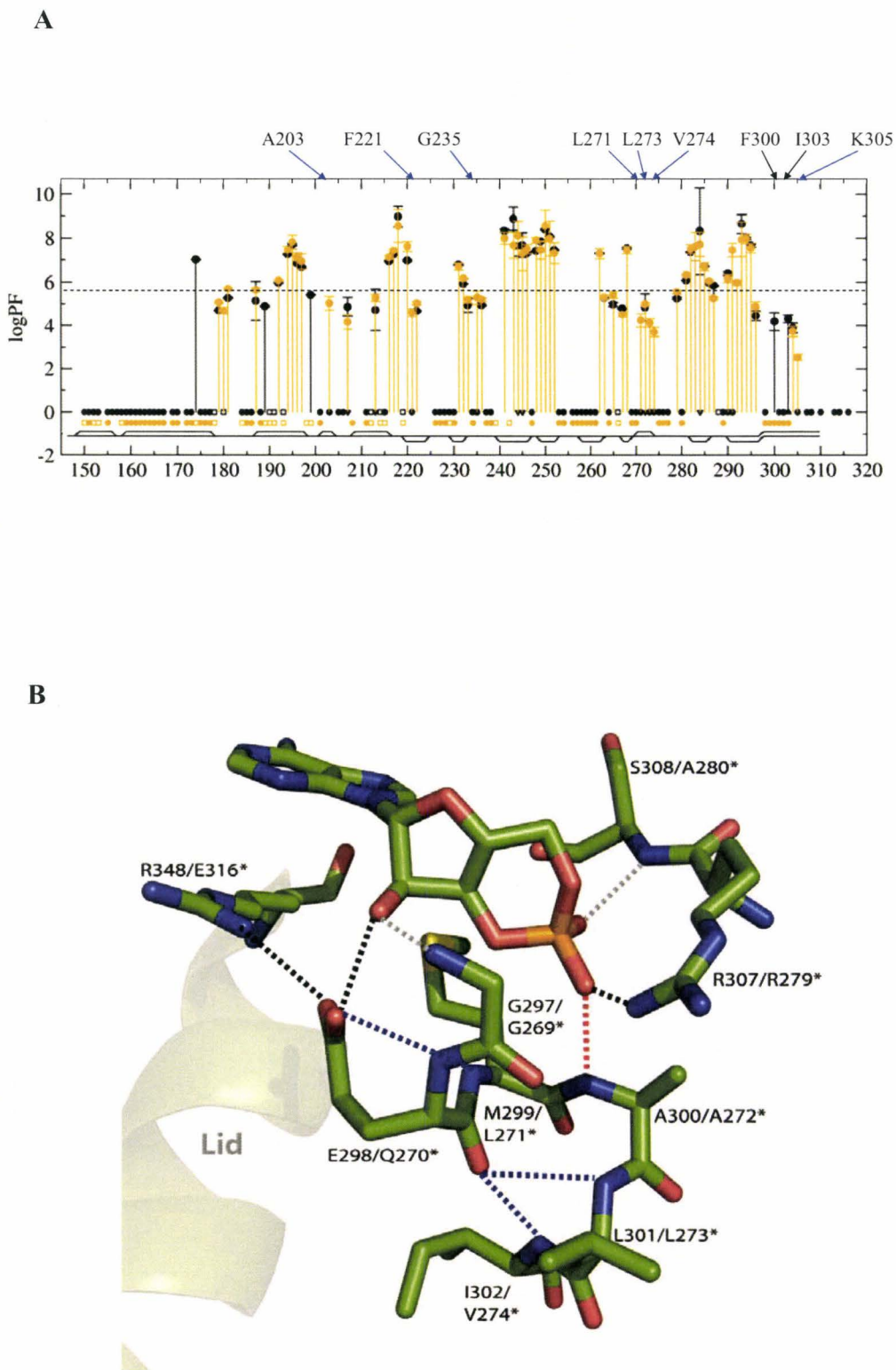
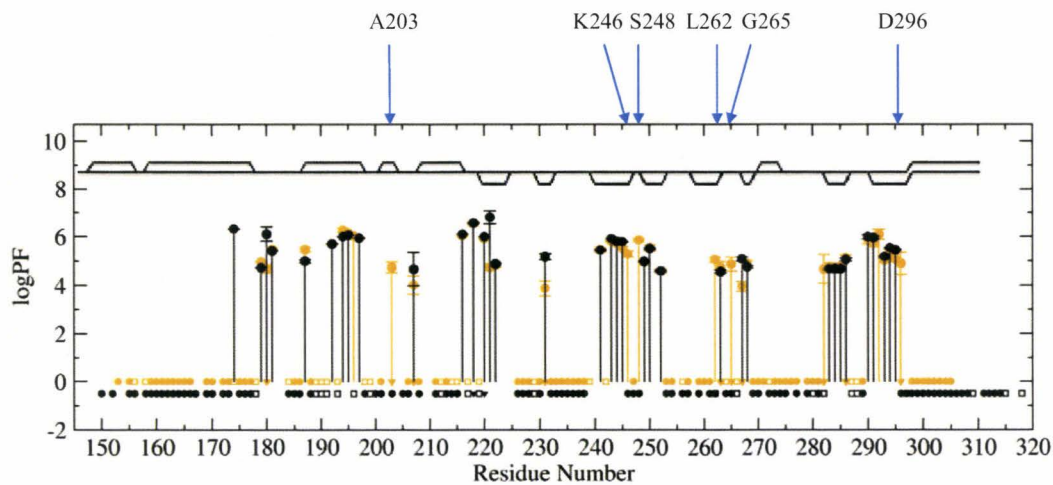


Figure 3

A



B

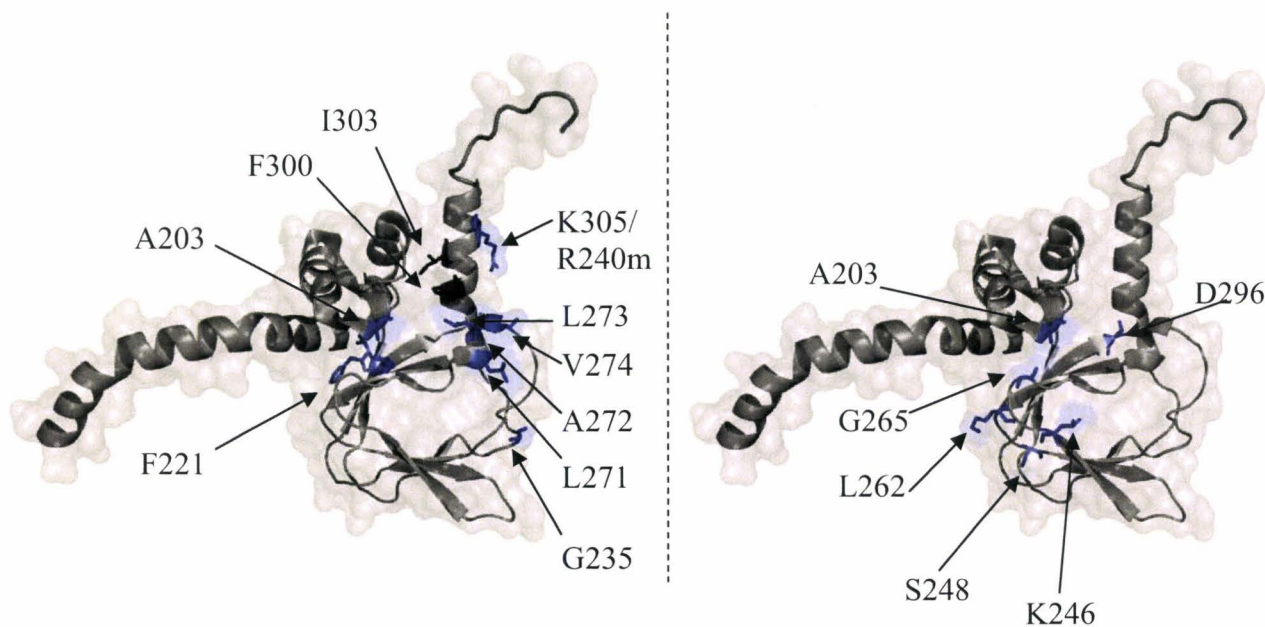
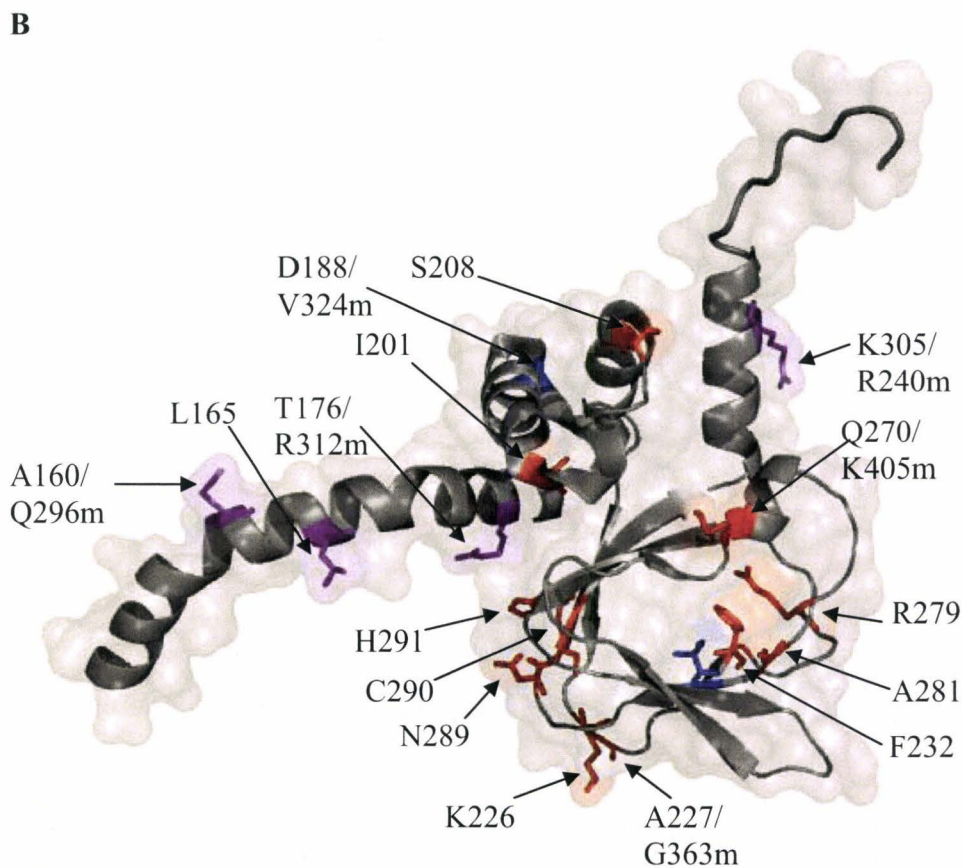
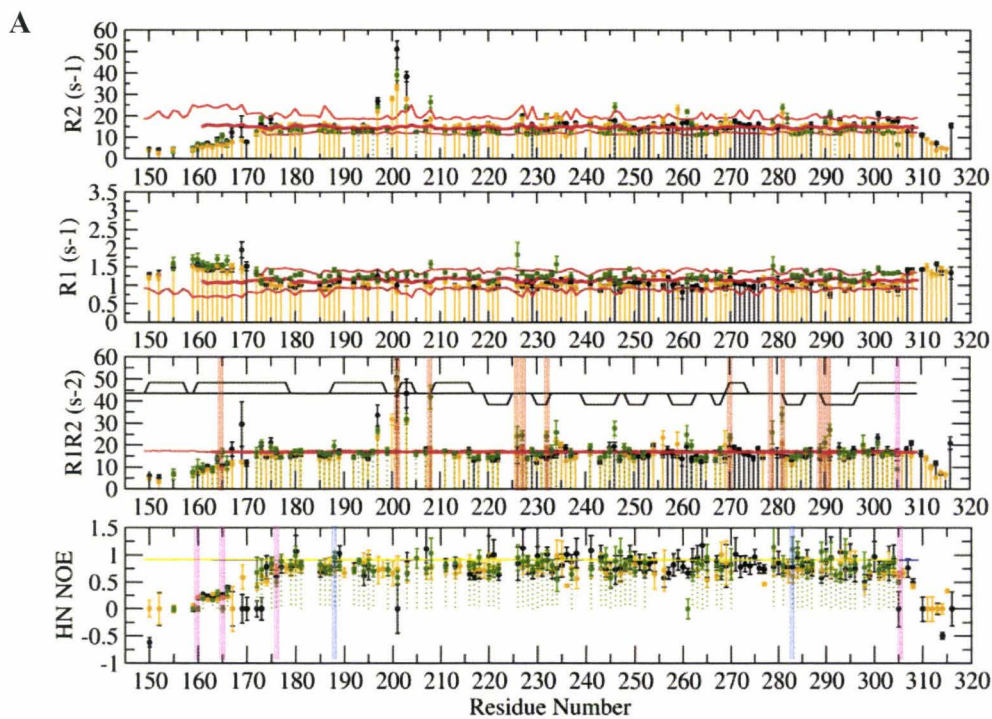
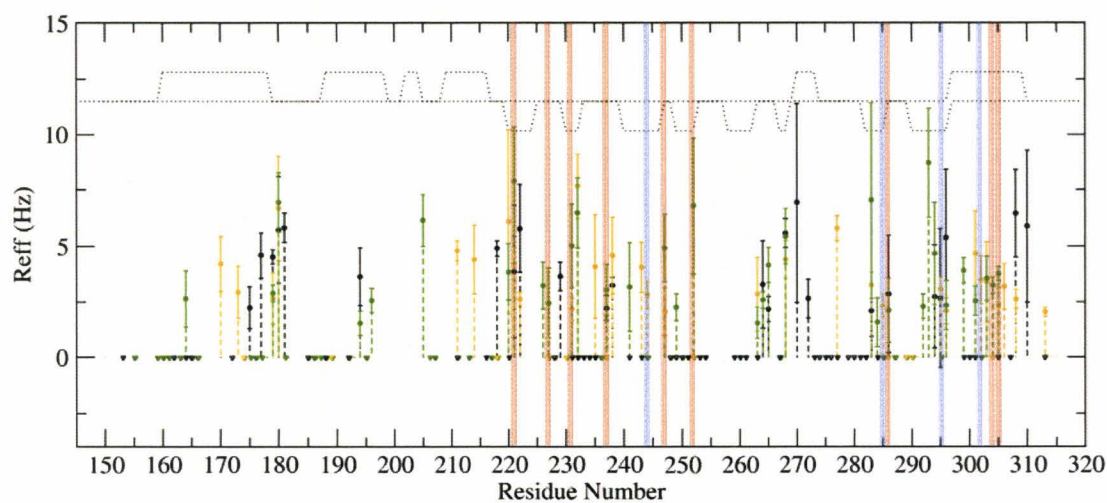


Figure 4



A



B

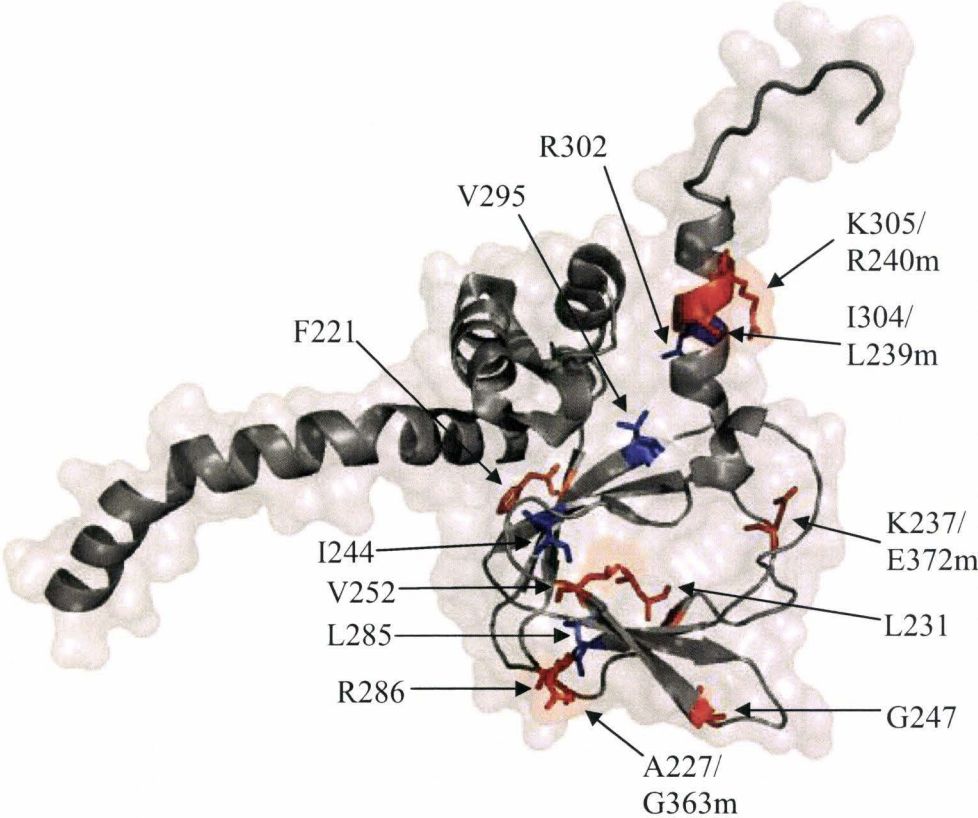
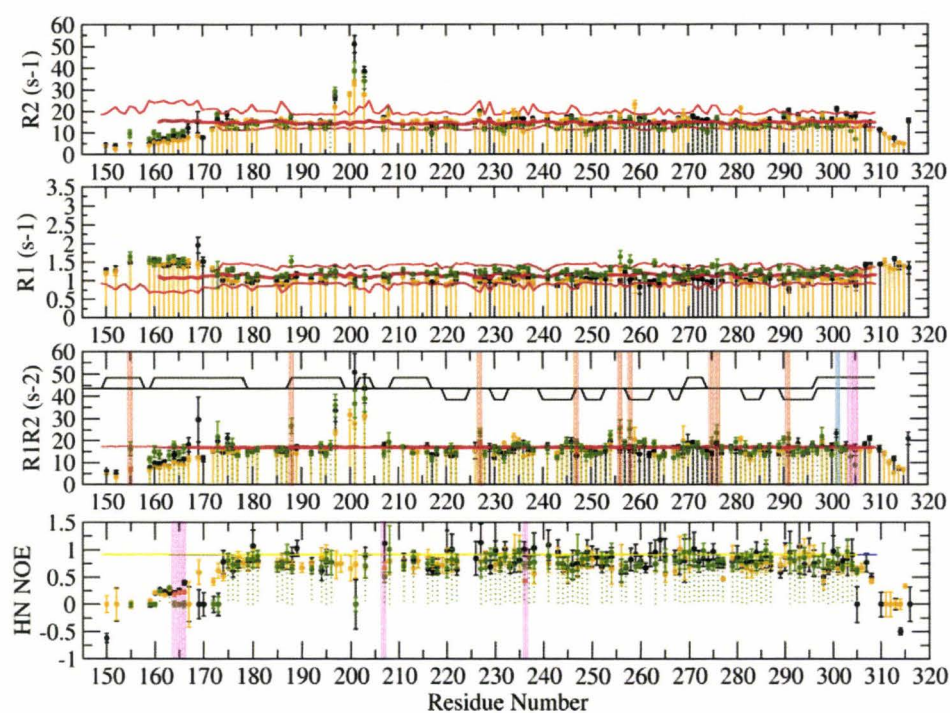
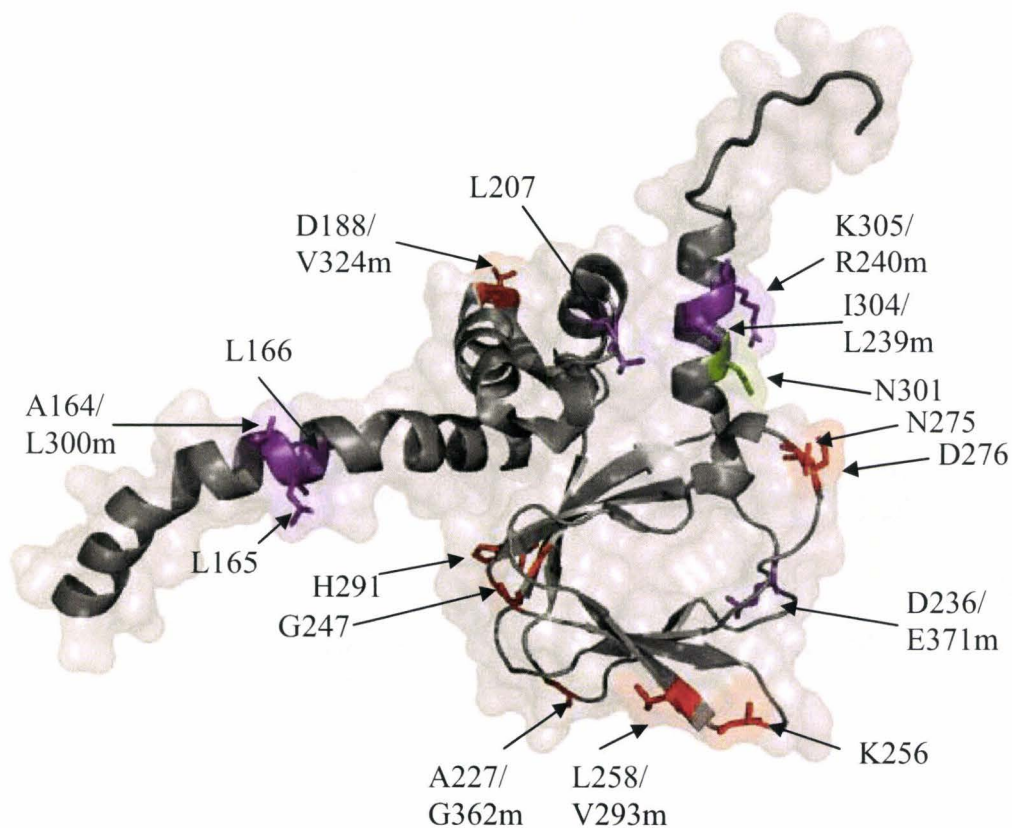


Figure 6

A**B**

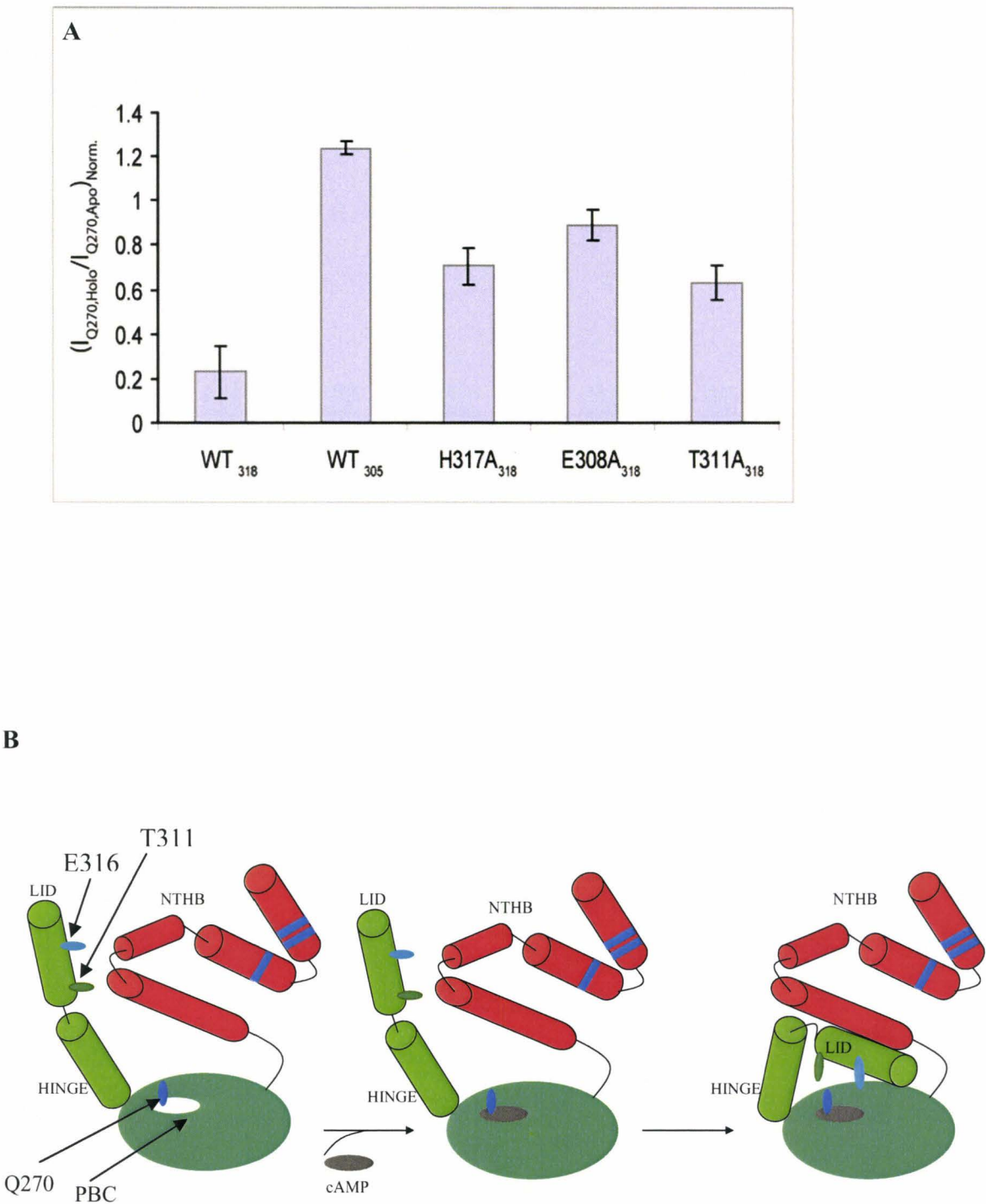
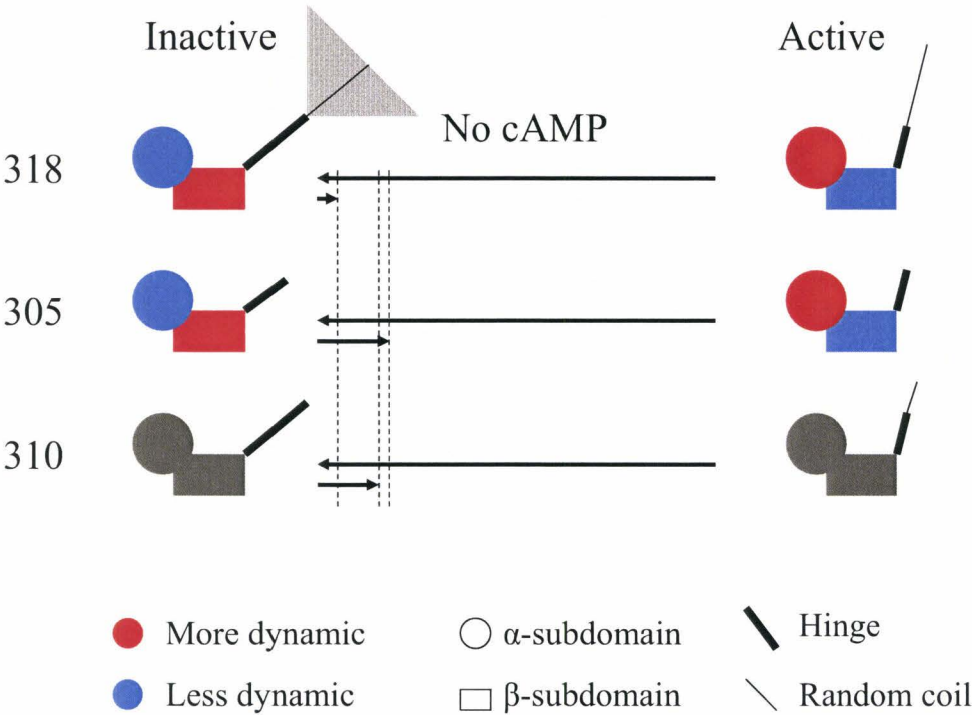
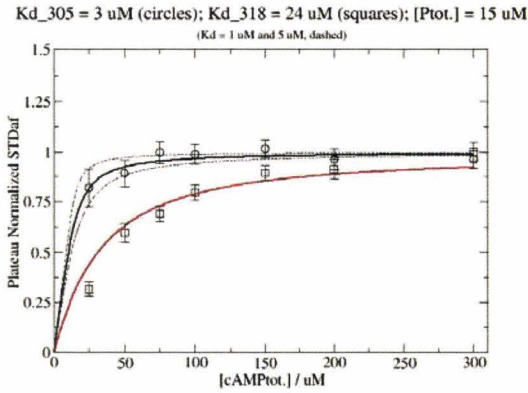


Figure 8

A



B



C

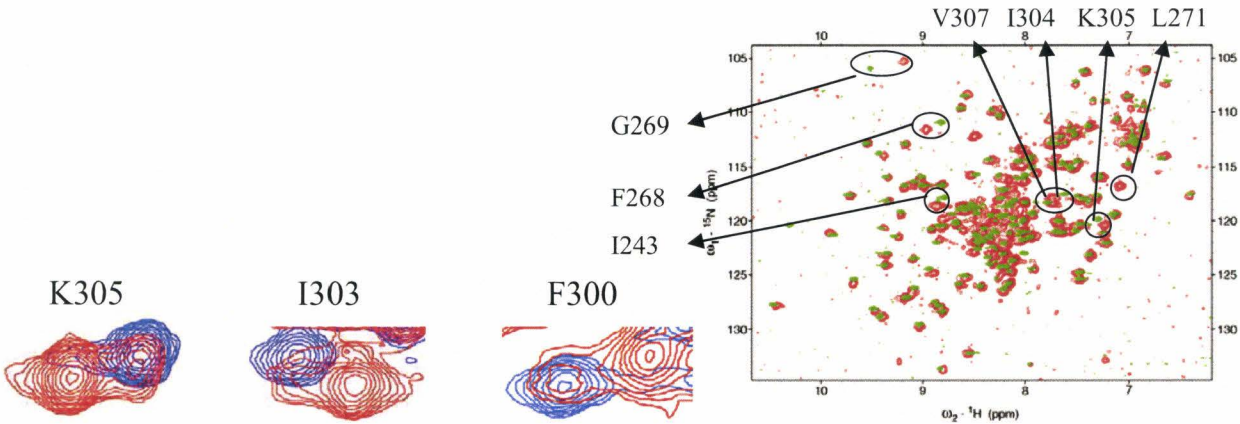


Figure 9

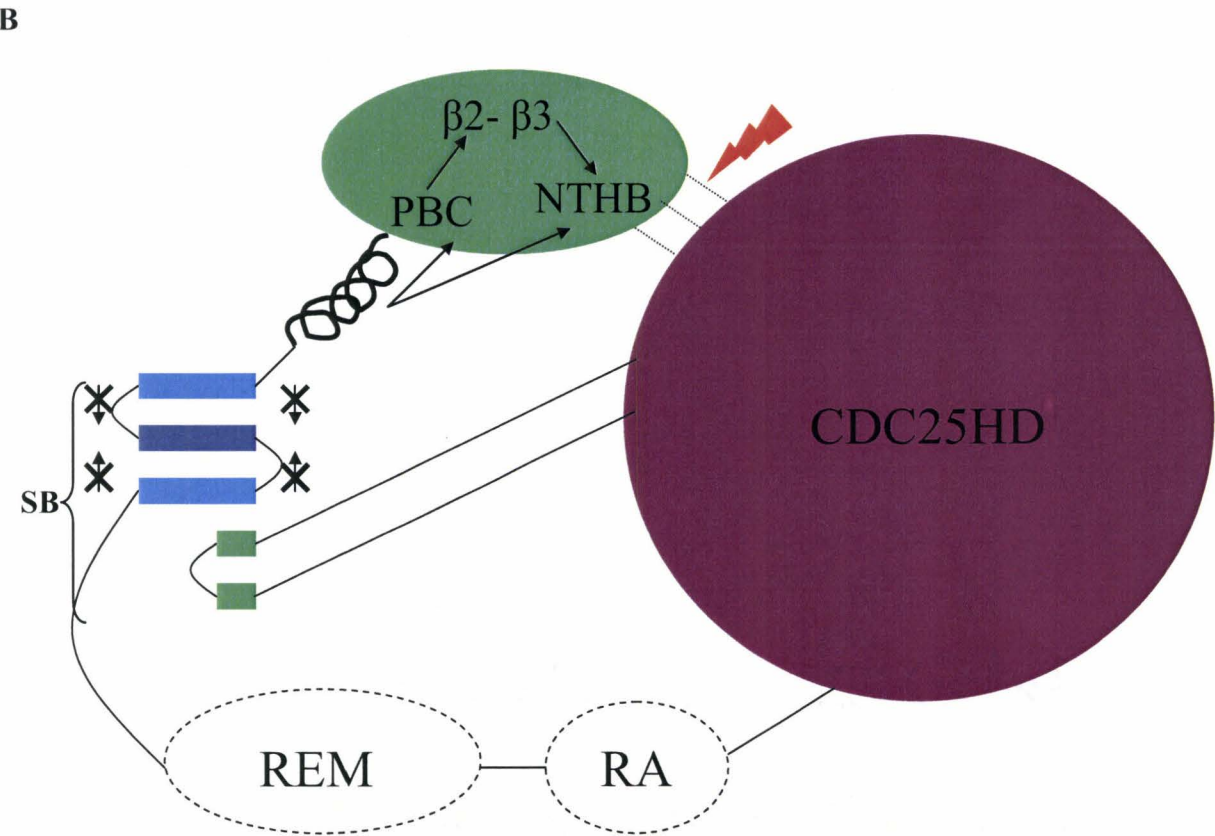
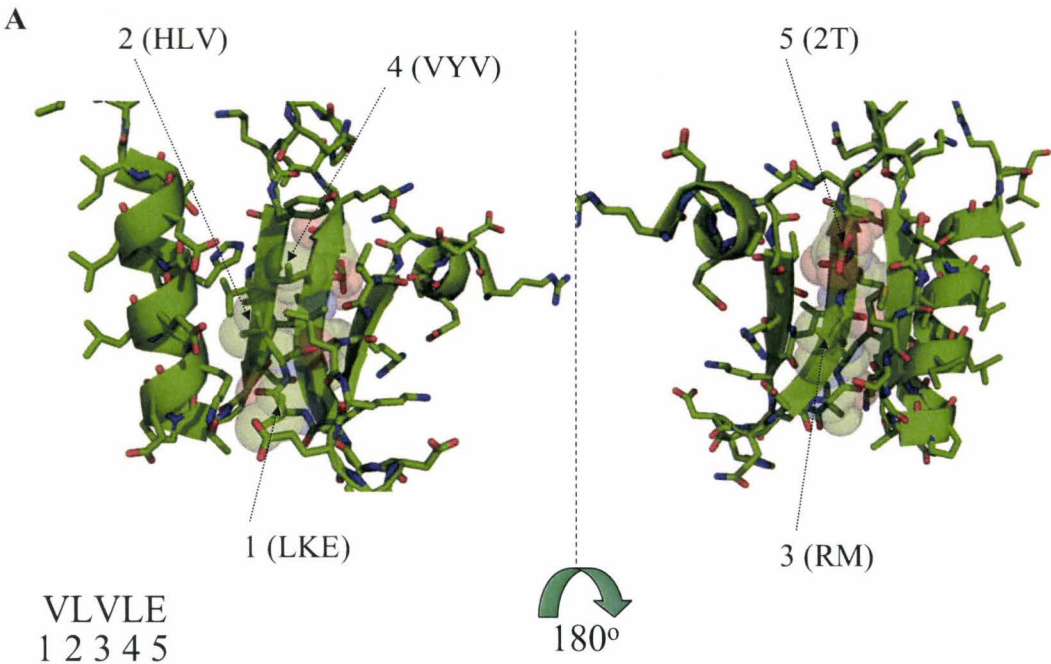


Figure 10

5.1 Conclusion:

5.1.1 What have this study accomplished? We investigated the response of human Epac1 CBD toward cAMP in three stages. In the first stage, we mapped the intra-molecular allosteric pathway of cAMP from its distal binding site in the PBC to the sites relevant in the steric inhibition of the Epac exchange activity, including the NTHB. Combination of cAMP mediated chemical shifts, secondary structure probability and hydrogen-deuterium exchange perturbations lead to the identification of the C-terminal Hinge helix and the $\beta 2$ - $\beta 3$ region as allosteric hot spots that relay the ligand binding information to the ionic interface between the RR:CR for the release of the steric inhibition (1). We identified that the L273-F300 hydrophobic bridge between the Hinge and the PBC is part of a significantly extended interaction network that is not only confined to hydrophobic contacts, but also contains ionic/polar interface mediated primarily by K297 in the N-terminus of the Hinge helix and the A272 and D276 in the PBC helix-loop motif. R279 and A280, in this motif, were also identified to mediate the communication between the PBC and the $\beta 2$ - $\beta 3$ regions. Based on the structure of the ligand free state of Epac2, we hypothesize that the Hinge helix is mainly communicating with the $\alpha 3$ - $\alpha 4$ and the $\beta 2$ - $\beta 3$ region can contact the $\alpha 2$ and possibly the $\alpha 1$ helix in the NTHB. Based on our data, we also suggest the presence of local fluctuations in the two key allosteric hot spots, the Hinge and $\beta 2$ - $\beta 3$ regions, which co-occur with local fluctuations in the N-terminus of PBC in the cAMP bound state (1). Recent sequence and spatial based investigations of conserved motif within known CBDs, have also identified the Hinge helix and the $\beta 2$ - $\beta 3$ loop as evolutionary conserved motifs in the cAMP sensing domains (2, 3).

In the second part of this study, inspired by the lack of major structural response of the ionic latch region in the NTHB toward cAMP, we decided to investigate the possible role

of dynamic as a relay vehicle for the allosteric free energy of cAMP to the RR:CR interface. Hence we measured the backbone ^{15}N relaxation of Epac1 CBD and indeed the role of entropy in the weakening of the RR:CR ionic interface was confirmed. Upon ligand docking, the PBC and the $\beta 2$ - $\beta 3$ loop quench simultaneously in both ps-ns and μs -ms time scales, further confirming the positive cooperatively between these two elements in both ligand recognition and allostery. For the Hinge helix we observed mixed pattern of dynamics in which the patches with increased and decreased entropy were observed for the C- and N-termini of the helix respectively. The dynamics enhancement (flexibility) of the C-terminus of the Hinge was consistent with the observed destabilization of this segment upon cAMP binding, as seen by the secondary chemical shifts, and may facilitate the rigid body rotation of the RR away from CR as proposed based on the mutational analysis (4). As a matter of fact the Hinge helix is followed by the switch board region, which is proposed to be the key pivotal point for the cAMP mediated rigid body rotation (4). The cold patches map to the N-terminus of the Hinge helix that is presumably in close proximity of the core of the β -subdomain in the cAMP bound state (4). The mixed pattern of dynamics observed for the $\alpha 4$ helix in the NTHB facing the Hinge helix can also be explained by release and formation of new contacts via the re-arrangement of the Hinge helix as discussed in chapter 3. The ionic latch region spanning $\alpha 1$ - $\alpha 2$ in the NTHB, displayed marked increase in ps-ns and μs -ms time scales dynamics upon cAMP binding. This indicated the existence of a negative cooperatively between the ionic latch and the β -barrel containing the cAMP binding site. Hence, upon ligand binding, the entropic cost of the maintenance of RR:CR ionic interface increases and this can further facilitate the

removal of the RR away from the CR as the result of the cAMP mediated Hinge rotation (11).

The third stage of this study, investigated the role of the C-terminal Hinge-Lid region (CHLR) in the cAMP recognition and allostery in human Epac1 CBD. The primary reason for the selection of this motif was the observation based on the secondary chemical shift data that indicated the formation of a helix-loop-transient helix conformation spanning the complete Hinge-Lid region (D296-G318) upon cAMP binding. In all known structures of human CBDs, it has been shown that a residue will stack against the base of the cAMP to provide protection against solvent and phosphodiesterases (5). In Epac several residues (T311 (6), L314 (7) and H317 (6)) are proposed to form this stacking interaction and they all map to the Lid region. However, recent investigations have also suggested that these residues, such as H317 (8), might not form a proper stacking contact with the ligand and in contrast interact with the N-terminus of the PBC (N-PBC), where the phospho-ribose moiety of the cAMP binds. The K305-G318 segment of the Hinge-Lid region marks the loop-transient helix motif and this motif might be able to bend toward the PBC and provide stable/transient interaction with the N-PBC/cAMP. Hence, we investigated in details two Epac1 CBD constructs; Epac1 149-305 and Epac1 149-318, in order to identify the CHLR effects on the ligand recognition and allostery.

Based on our investigations on the two CBD constructs, we proposed that CHLR attenuates cAMP mediated Epac activation at both levels of ligand recognition and allostery. The effects of CHLR are categorized in two groups; cAMP free and bound states.

In the absence of both the ligand and CHLR, the N-terminus of the Hinge helix was in a conformation closer to that of the bound, with a small population in $\sim$ ms exchange

between the two conformers. This triggered the entire conformation of CBD to adopt a pseudo-cAMP like conformation. This indicates that first, N-terminus of the Hinge helix (which still retains its helix conformation after cAMP is bound) has an intrinsic tendency to move toward PBC and second the Hinge helix alone can communicate, directly or indirectly, with all cAMP-sensitive regions in the Epac CBD. Hence, the CHLR further distances the Hinge helix from the PBC in the absence of cAMP and the conformation resembles less that of the cAMP bound state.

In the presence of cAMP, we observed multiple evidences of the inhibitory effects of CHLR at both the levels of kinetic and thermodynamics. CHLR reduced the affinity of the CBD toward cAMP ~six times and perturbed the hydrogen binding network at the N-PBC. CHLR also reduced the entropy and increased the enthalpy of the cAMP bound conformation, hence disfavoring this state thermodynamically. We proposed that the negative cooperatively of CHLR in the cAMP mediated activation is at least in part mediated via Q270 at the N-PBC, which has been previously shown to disfavor the active state in Epac CBD (6). Indeed the presence of CHLR, induced slow ms dynamics in this residue, which were quenched upon CHLR deletion.

5.1.2 What are the impacts of this study? The primary contribution of this investigation stems from the similarities and differences identified between the Epac and PKA systems. In terms of intra-molecular allosteric pathways both systems are similar in the use of the two allosteric hot spots; the C-terminal Hinge helix and the $\beta 2$ - $\beta 3$ loop (1, 9). Perturbations at the distal PBC and BBR sites in both CBDs are reported indirectly to the NTHB via the two allosteric sites, which then mediate the unmasking of the catalytic subunit/region in PKA/Epac. It is possible that the two hot spots might even be a more

generalized feature based on sequence and spatial alignments of different CBDs (2, 3). However, two key differences between Epac and PKA were observed in the recognition of cAMP and cAMP mediated control of the RR:CR interface.

According to the cAMP bound crystal structure of the PKA CBD, both ends of the PBC form a stable backbone to cAMP hydrogen bond via G199 and A210, corresponding to G269 and A280 in Epac (10). These two inter-molecular contacts tend to constrict the PBC conformation. In solution, the two aforementioned hydrogen bonds presumably remain tight in PKA CBD due to protection from D₂O exchange (9). However this is not true in the case Epac, suggesting a more relaxed/open PBC conformation (1). This could partially explain both the lower affinity of Epac toward cAMP and its ability to accommodate more bulky cAMP analogs (6). A key difference also exists in the cAMP mediated control of the NTHB between the two systems. In the PKA CBD, most residues within this region display minimal protection from D₂O exchange and are not affected via cAMP (9). However the NTHB of Epac CBD possesses several residues with maximal protection, which is enhanced upon cAMP binding and responds concertedly to the quenching of the global unfolding conferred via cAMP binding (1). This indicates that the group of residues that are sensitive to the cAMP-dependent global control of the unfolding events, is much more extended in Epac CBD than in PKA, which only confines it to its β -subdomain, and suggested a tighter inter-subdomain coupling in Epac CBD (1). This is also consistent with our observation of higher cooperativity of unfolding in Epac than in PKA. It is possible that the cAMP modulated stability of the NTHB might have survived evolutionary selection due to its critical role in the cAMP mediated activation of Epac exchange activity (1).

The dynamic landscape of both CBDs of Epac and PKA were similar with positive cooperativity between PBC and $\beta 2$ - $\beta 3$ loop and negative cooperativity between the two subdomains of each CBD in respond to cAMP binding (11, 12). This highlighted the conserved role of entropy in the mechanism of activation of PKA and Epac and is likely to represent a generalized mechanism of allostery in cAMP responsive proteins (11). Similar suggestions in the role of entropic contributions to allostery in the absence of significant structural alternations have been previously provided at the level of both experimental observations (13) and theory (14).

Identifications of the similarities and differences in ligand recognition and allostery between the two ubiquitously expressed Epac and PKA is crucial in the generation of cAMP analogs that can selectively recognize and activate these global cAMP receptors in the human body.

5.1.3 What are the short comings of this study? There is room for improvement in our attempt to investigate cAMP signaling in the human Epac1 CBD. In mapping the intra-molecular allosteric pathway of cAMP, we relayed on the backbone as a probe of cAMP-induced perturbations. This allows for the identification of backbone to backbone and to some extent side chain to backbone interactions. However, it is well known that a significant portion of intra-molecular communication within the proteins is mediated via side chain-side chain contacts. For example, in the case of Epac1 CBD, in chapter 2 we proposed that the side chain of F300 in the Hinge and W241 in the $\beta 2$ - $\beta 3$ region can transmit the cAMP signal to the NTHB. In order to further investigate this transmission pathway, the atoms in the side chains of these residues need to be assigned and be analyzed in terms of chemical shifts and dynamic perturbations upon ligand binding. A complete

side chain assignment allows one not only to investigate the perturbations in the side chains but also gain geometric insight of the cAMP mediated structural rearrangements by measurements of NOEs between different atoms to identify inter-atomic distances and their change upon cAMP binding.

The second improvement in this study can be the use of longer constructs that span the entire Lid region. Although the majority of the proposed interactions between the Lid and PBC/cAMP are within 310-317 segment (6, 7)), which is spanned by the Epac1 149-318 constructs, it is possible that the potential contribution of the Lid to the CBD ligand recognition and allostery is to some extent affected as the result of C-terminal truncation. We have preliminary results of a promising expression and solubility of a longer Epac construct, Epac1 149-329, that contains eleven residues C-terminal to 318 (Figure 1a). According to molecular simulations for the isolated CBD of Epac1 149-329, it has been suggested that in the cAMP bound form a helix will form at the C-terminus of this construct and will extend up to E325 (7). According to our secondary chemical shift data on Epac1 149-318, we can observe a transient helix at the C-terminus of the CBD (Chapter 2, Figure 2c), which might correspond to the same helix in the simulation. Formation of this helix might stabilize the Lid region and its contribution to ligand recognition and allostery. This construct will also be a good window for looking into the base modified cAMP analogs, which are proposed to effect the mode of Lid interactions (6). We also have promising preliminary data for a well-expressed Epac construct, Epac1 149-472, that contains the CBD and the REM domain of Epac (Figure 1b). This construct has been shown experimentally to contain all parts of Epac1 that respond to cAMP (7), as monitored by mass spectroscopic based HD exchange in the Δ DEP-Epac1 (149-881). Numerous

attempts to purify this protein have failed yet, primarily due to formation of inclusion bodies upon French Press, even when it is tagged to GST. Refolding attempts have managed to rescue this construct as a soluble protein (cleaved from GST), however preliminary NMR data suggest that the refolded protein does not retain a proper 3D structure (Figure 1b). At the moment we are trying to purify this construct in the presence of sarcosine that might prevent the construct to form inclusion bodies.

Another improvement could be the utilization of a de-coupler mutant, *i.e.* L273W in the PBC, which is known to decouple cAMP binding from Epac activation (4), in the testing of the Hinge intrinsic tendency to move toward PBC. In chapter 4, we suggested the existence of such a tendency, by comparison of the Epac 149-305 and Epac 149-318 constructs. Trp in the place of L273 is proposed to block the inward movement of the Hinge helix by steric clash with the F300 in the Hinge helix (4). If Hinge conformation in the Epac 149-305 construct in the absence of cAMP is already close to the bound conformation, then L273W should provide a steric clash potential to move the Hinge away from the PBC. If this hypothesis is correct, then we should observe more similarities between the apo L273W Epac 149-305 and apo wild type Epac 149-318 constructs than the wild type-wild type comparison in the apo forms.

It is also possible to take advantage of the two well characterized cAMP analogs; 2'-OMe-cAMP and 8-pCPT-2'-OMe-cAMP that are known to engage the Lid in the Epac activation process via mutational investigations (6). Both analogs are proposed to communicate with the Lid via 2'-OMe group and as mentioned in Chapter 4, Q270, which disfavors the cAMP bound conformation, favors the 8-pCPT-2'-OMe-cAMP bound state. It is possible that the Q270 and the 2'-OMe group act synergistically to communicate with

the Lid. The change in the nature of interactions between the analogs and the PBC convert Q270 from an attenuator to an inducer of Epac activation (6). By comparative analysis of Epac 149-305 and Epac 149-318 affinities toward these Lid engaging analogs, we might be able to validate, to a certain extent, that the Lid in the latter construct is able to form interaction with N-PBC (Q270) / 2'-OMe group. We observe a 6 fold reduction in affinity for cAMP upon addition of CHLR. If the 2'-OMe group can, in parallel with Q270, communicate with the Lid, then the observed reduction in affinity for ligand binding between the two CBD constructs is expected to be less severe for these two analogs compared to cAMP. The primary purpose of this comparative affinity analysis would be to validate the ability of the Lid to make contact with N-PBC/2'-OMe group even in the presence of C-terminal truncation effects.

Precise elucidation of the mechanism of negative cooperativity conferred by CHLR, is crucial to our understanding of Epac allostery and may further highlight differences between the two globally expressed systems of Epac and PKA in human body. This could potentially culminate in the generation of new Epac specific analogs for the treatment of diseases ranging from Alzheimer to diabetes. For a better picture of the mechanism of negative cooperativity, it would also be beneficial to consider an Ala scan of the CHLR region to try to pin point residues that might contribute to the negative effects on ligand recognition and allostery.

5.2 Acknowledgments:

I would like to thank Ali Alavi for his assistance on the preparation of Epac1 149-329 construct.

5.3 References:

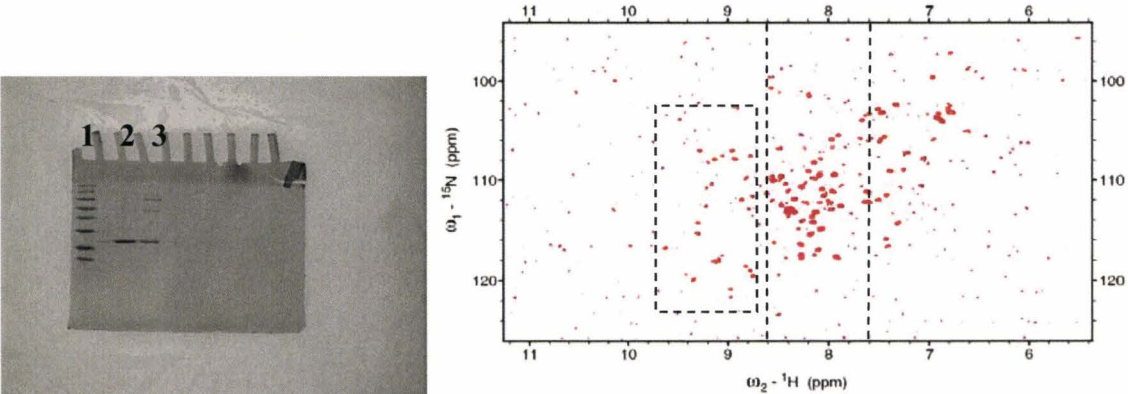
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5.4 Figure captions:

Figure 1) sample preparation of longer Epac1 CBDs. a) Preparation of Epac1 149-329 with the conventional protein preparation protocol for GST tagged proteins as described in chapter 2. In the gel shown; lane 1, 2 and 3 are ladder, purified protein from first and second elution respectively. To the right the HSQC spectrum of this protein in the presence of cAMP is shown. The two dashed lines represent the range within which the signals from unstructured parts of the protein are expected to be and the part in the dashed rectangle is the region with dispersed signal, representative of structured regions within the protein. b) The top left panel is the gel for the expression check of four identical *E. coli* colonies expressing Epac1 149-472 induced with IPTG (+). The top right panel is the fractions of ion exchange purified Epac1 149-472. The proteins were unfolded and refolded with urea after French Press, then bound to glutathione beads following cleavage with thrombin and ion exchange. The lower panel is the HSQC spectrum of Epac1 149-472 bound to cAMP. As can be seen most of the signal are within 7.8-8.5 ppm representing mostly unfolded structure. The circles on the spectrum are signals from the side chains.

A



B

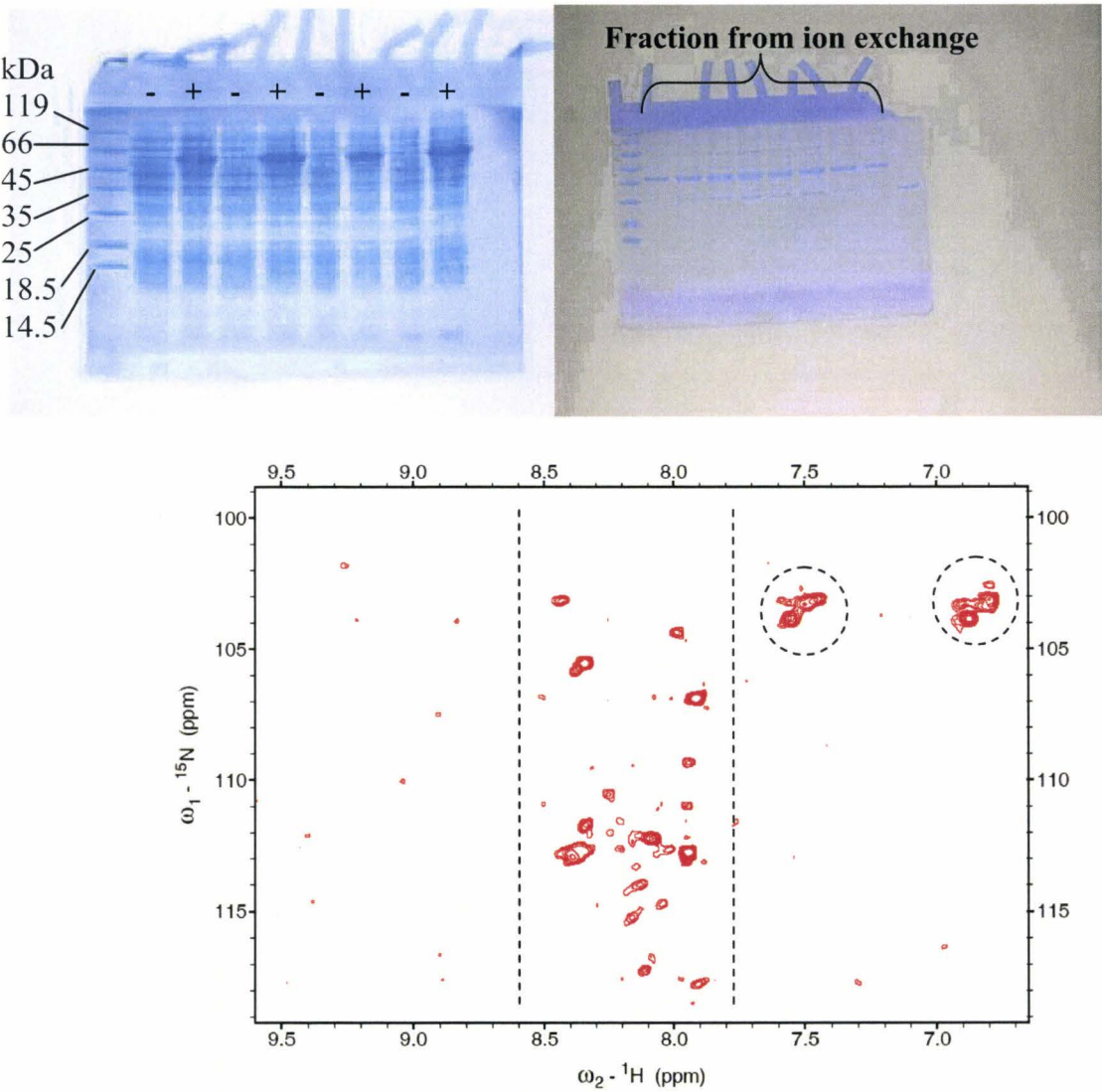


Figure 1