Cyclic AMP-induced Conformational Changes in Mycobacterial Protein Acetyltransferases

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Background: cAMP allosterically regulates protein lysine acetyltransferases in mycobacteria.

Results: cAMP binding alters dynamics of the cyclic AMP binding domain and the interdomain linker in these proteins.

Conclusion: cAMP action is mediated by increased ordering upon binding without intricate allosteric networks.

Significance: Effects mediated by cAMP in these proteins are distinct from relays seen in cAMP binding proteins characterized thus far.

The activities of a number of proteins are regulated by the binding of cAMP and cGMP to cyclic nucleotide binding (CNB) domains that are found associated with one or more effector domains with diverse functions. Although the conserved architecture of CNB domains has been extensively studied by x-ray crystallography, the key to unraveling the mechanisms of cAMP action has been protein dynamics analyses. Recently, we have identified a novel cAMP-binding protein from mycobacteria, where cAMP regulates the activity of an associated protein acetyltransferase domain. In the current study, we have monitored the conformational changes that occur upon cAMP binding to the CNB domain in these proteins, using a combination of bioluminescence resonance energy transfer and amide hydrogen/deuterium exchange mass spectrometry. Coupled with mutational analyses, our studies reveal the critical role of the linker region (positioned between the CNB domain and the acetyltransferase domain) in allosteric coupling of cAMP binding to activation of acetyltransferase catalysis. Importantly, major differences in conformational change upon cAMP binding were accompanied by stabilization of the CNB and linker domain alone. This is in contrast to other cAMP-binding proteins, where cyclic nucleotide binding has been shown to involve intricate and parallel allosteric relays. Finally, this powerful convergence of results from bioluminescence resonance energy transfer and hydrogen/deuterium exchange mass spectrometry reaffirms the power of solution biophysical tools in unraveling mechanistic bases of regulation of proteins in the absence of high resolution structural information.

The second messenger cyclic adenosine 3’,5’-monophosphate (cAMP) mediates its actions by binding to proteins containing cyclic nucleotide binding (CNB)5 or GAF (cGMP-specific phosphodiesterases, bacterial adenyl cyclase, and the bacterial FhLA transcriptional regulator) domains (1, 2). CNB domains that bind cAMP are found coupled to diverse effector domains, such as cyclic nucleotide-regulated ion channels, catabolite activator protein (CAP, or cAMP-responsive proteins), guanine nucleotide exchange proteins activated by cAMP (Epac), and cyclic nucleotide-dependent protein kinase regulatory subunits. CNB domain-containing proteins are found in both prokaryotes and eukaryotes and are easily identifiable by a 12-amino acid motif, referred to as the phosphate binding cassette (PBC), containing invariant residues critical for anchoring cAMP. Indeed, the structural biology of diverse CNBs reveals a highly conserved architecture with an α-subdomain and a β-subdomain comprising an eight-stranded β-sheet containing the PBC. The PBC provides a conserved high affinity binding pocket for both cAMP and cGMP (1, 3). cGMP-binding proteins contain a Ser/Thr residue following a conserved Arg, as opposed to an Ala residue found in cAMP-binding proteins (4).

The allosteric regulation of various proteins that contain cAMP-binding domains has been elucidated in extensive detail at the structural level. Crystal structures of the cAMP-dependent protein kinase (PKA), CAP, cyclic nucleotide-regulated channels, and Epac1 reveal interactions of cAMP with the PBC as well as additional conformational changes that are relayed from the CNB domain to the associated effector domains. These studies have suggested that all CNB domains regulate the effector domain upon binding of cAMP through hydrophobic interactions (5). CNB domains have been shown to adopt two

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5 The abbreviations used are: CNB, cyclic nucleotide-binding domain; AT, acetyltransferase; BRET, bioluminescence resonance energy transfer; CAP, catabolite activator protein; Epac, exchange protein activated by cAMP; HDXMS, amide hydrogen/deuterium exchange mass spectrometry; KATms, MSMEG_5458 lysine acetyltransferase; KATmt, Rv0998 lysine acetyltransferase; (R)n-cAMPS, adenosine-3’,5’-cyclic monophosphorothioate, (S)n-cAMPS, adenosine-3’,5’-cyclic monophosphorothioate, (S)n isoform; USP, universal stress protein; bis-Tris, 2-[bis(2-hydroxy-ethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; PBC, phosphate binding cassette.
cAMP-mediated Regulatory Switches in Mycobacteria

stable end point conformations, denoted as the B (cAMP-bound, active; supplemental Fig. 1A) (6) and the H (cAMP-free, inactive) conformation, where large changes resulting from repositioning of the C-helix to which the effector domain is attached are observed. Conserved residues in the PBC are critical in anchoring the different functional moieties in cAMP and present a high affinity binding pocket that combines precise geometry with specificity (supplemental Fig. 1A). These include a conserved Gly, whose backbone amide nitrogen along with the side chain of a Glu residue anchor the ribose 2′-OH. A conserved Arg anchors the phosphodiester bond, and an aromatic residue, part of a “lid” region, stacks against cAMP and stabilizes its binding. A “hinge” region consisting of hydrophobic residues is responsible for propagating the effects of cAMP binding to distal regions of the protein (reviewed in Ref. 7).

CAMP-dependent dynamics has also been studied by amide hydrogen/deuterium exchange coupled to mass spectrometry (HDXMS), a powerful technique to probe dynamics of proteins in solution (8). These studies on different classes of CNB domains (9–12) have provided important insights into cAMP-mediated allostery. The cAMP binding site and contacts are highly conserved, as are a number of allosteric relays that couple cAMP binding to distal functional regions of the protein. In Epac, this is believed to involve the hydrophobic hinge (13), and in PKA, an electrostatic relay between the Arg of PBC and a conserved Asp (10, 14, 15) is important. Recently, we have shown that the basis for CAMP action in the Regulatory (R) subunit of PKA was a combination of “induced fit” (10) and “conformational selection” (6). In the induced fit model, conformational changes following CAMP binding are propagated throughout the entire protein, whereas the conformational selection model assumes that CNB domains are metastable and populate all stable end point conformations, one of which is preferentially “selected” by cAMP. The importance of allosteric relays for CAMP action is highly relevant to both models. Similar mechanisms have been described with the CNB domains in Epac, where evidence of the apo-CN domain existing in an ensemble of two or more conformations provided the primary basis for conformational selection (16).

Mycobacteria, including Mycobacterium tuberculosis, which is the causative agent for tuberculosis, contain an abundance of proteins involved in CAMP metabolism (17, 18). Genome analysis followed by biochemical studies has revealed that a number of enzymes are capable of synthesizing and regulating CAMP levels in these bacteria under different environmental conditions (19). Interestingly, only a single CAMP phosphodiesterase has been characterized so far (20, 21), but the ability of mycobacteria to secrete large amounts of CAMP (19) suggests that secretion, rather than hydrolysis of CAMP, may be a more efficient means of regulating intracellular CAMP. The importance of secreted CAMP in modulating macrophage response to M. tuberculosis has been described (22), and elevated levels of CAMP of bacterial origin are observed in macrophages following infection by mycobacteria (23, 24). The high levels of CAMP found in both pathogenic and non-pathogenic mycobacteria, however, suggest that basic cellular processes are regulated by CAMP in these bacteria. CAMP-binding transcription factors, with some similarity to the CAP from Escherichia coli, have been characterized and found to regulate a number of genes (25–28). We have recently described novel proteins that contain a CNB domain fused to an acetyltransferase (AT) domain (29). These proteins, the products of the MSMEG_5458 gene from Mycobacterium smegmatis and the rv0998 gene from M. tuberculosis, contain an N-terminal CNB domain fused to a C-terminal domain that is similar to the GNAT (GCN5-related N-acetyltransferase) family of enzymes (29). Importantly, these mycobacterial proteins are able to acetylate the ε-amino group of lysine residues in a CAMP-regulated manner (29, 30). This domain organization is unique to mycobacteria and therefore provides an opportunity to monitor conformational changes that occur upon cAMP binding to a novel effector domain, namely a protein acetyltransferase.

In this study, we monitor the dynamic conformational changes that occur upon CAMP binding to MSMEG_5458 (henceforth referred to as KATms) using bioluminescence resonance energy transfer (BRET) (31) as well as HDXMS. CAMP binding to full-length KATms induced a conformational change that could be detected by an increase in BRET. Employing HDXMS, we observed that in contrast to other CNB domains, no CAMP-dependent changes in deuterium exchange in KATms were observable in regions associated with allostery in other CNB domains. Instead, we show the importance of a putative helical region between the CBD and the AT domain and identify two residues that are crucial for transmitting the information of CAMP-binding to the C-terminal AT domain. This therefore represents the first example of a primitive CNB domain lacking specific allosteric relays that couple CAMP binding to regulation of an associated catalytic domain.

EXPERIMENTAL PROCEDURES

All fine chemicals were from Sigma-Aldrich. Routine bacterial growth medium (Luria-Bertani) and Terrific Broth were purchased from Colloids Impex (Bangalore, India). Restriction enzymes were from MBI Fermentas (Burlington, Canada) or New England Biolabs. TALON metal affinity resin was from Clontech (Mountain View, CA), and glutathione-Sepharose was procured from GE Healthcare. Tritiated CAMP ([3H]cAMP) (specific activity 59 Ci/mmol) was obtained from PerkinElmer Life Sciences. Acetyl lysine antibody was obtained from Cell Signaling Technologies. Analogs of CAMP (B-bromoadenosine-3′,5′-cyclic monophosphorothioate, R₃, and S₃ isomers, and the non-brominated analogs) were from Biolog Life Science Institute (Bremen, Germany). The Porozyme-immobilized pepsin cartridge was from Applied Biosystems (Foster City, CA). Deuterium oxide (D₂O) and protein sequence analysis grade trifluoroacetic acid (TFA) were from Fluka BioChemika (Buchs, Switzerland).

Cloning and Mutagenesis—Primers used for cloning and mutagenesis (32) are detailed in supplemental Table 1. Constructs expressing wild type KATms and the R95K and E234A mutants have been described earlier (29). pPROKATms_p157A/p160A was generated using mutagenic primers MS5458P157A/P160A_1 and MS5458P157A/P160A_2. pGEX-KATmt_p160A/p163A was generated using mutagenic primers Rv0998P160A/P163A_1 and Rv0998P160A/p163A_1.
P163A_2. All mutations were verified by sequencing (Macrogen, Seoul, South Korea).

To generate the constructs used for BRET\(^2\) analysis, a region encompassing residues 1–333 of KATms was amplified using primers MS5458sensFLFWD and MS5458sensFLRVS, cloned into pBlueScript II KS(+) sequence, and then cloned into pGFP\(^{-}\)MCS-RLuc digested with EcoRV and XbaI to generate the plasmid pGFP\(^{-}\)-FL-Rluc. A construct encoding the CNB domain and the linker region (pGFP\(^{-}\)-CNBLIN-Rluc; encoding residues 1–215) was constructed similarly using primers MS5458sensFLFWD and MS5458sensCNBLINRVS.

Expression, Purification, and Characterization of Proteins—For biochemical assays, KATms and Rv0998 (KATmt) were purified essentially as described earlier using the cyc\(^{-}\) strain of \(E.\) \textit{coli} SP850, in order to obtain protein free of cAMP (29). For performing HDXMS, protein was further purified by size exclusion chromatography (S75 column, AKTA system, GE Healthcare) in buffer containing 50 mM Tris-HCl (pH 8.2), 1 mM DTT, 50 mM NaCl, and 10% glycerol. Purification of wild type and mutant proteins and cyclic nucleotide binding assays were performed as described earlier (29), and binding data were analyzed using GraphPad Prism 5.

\textbf{In Vitro BRET Assays—HEK 293T cells were transfected with pGFP\(^{-}\)-CNBLIN-Rluc or pGFP\(^{-}\)-FL-Rluc plasmids and, 72 h following transfection, lysed in 50 mM HEPES (pH 7.5), containing 2 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 10 mM sodium pyrophosphate, 80 \(\mu\)M glycerophosphate, 1 mM benzamidine, 1 \(\mu\)g/ml aprotinin, 1 \(\mu\)g/ml leupeptin, 5 \(\mu\)g/ml soybean trypsin inhibitor, 100 \(\mu\)M sodium orthovanadate, and 10% glycerol (31). Following brief sonication, lysates were centrifuged at 13,000 \(\times\) g, and the cytosol was removed.

\textbf{Apo-KATms} and USP were incubated first at 25 °C for 5 min. The reaction was then injected on to a chilled nano-UPLC sample manager following brief sonication, lysates were centrifuged at 13,000 \(\times\) g, and the cytosol was removed. The sample was washed through a 2.1 \(\times\) 30-mm immobilized pepsin column (Porozyme, ABI, Foster City, CA) with 10% glycerol (31, 32) to get a final pH read of 2.5. A 50-\(\mu\)l aliquot of the quenched sample (~200 pmol of protein sample) was then injected on to a chilled nano-UPLC sample manager (beta test version, Waters, Milford, MA) as described previously (33). The sample was washed through a 2.1 \(\times\) 30-mm immobilized pepsin column (Porozyme, ABI, Foster City, CA) with 10% glycerol (31, 32) to get a final pH read of 2.5. A 50-\(\mu\)l aliquot of the quenched sample (~200 pmol of protein sample) was then injected on to a chilled nano-UPLC sample manager (beta test version, Waters, Milford, MA) as described previously (33). The sample was washed through a 2.1 \(\times\) 30-mm immobilized pepsin column (Porozyme, ABI, Foster City, CA) with 10% glycerol (31, 32) to get a final pH read of 2.5. A 50-\(\mu\)l aliquot of the quenched sample (~200 pmol of protein sample) was then injected on to a chilled nano-UPLC sample manager (beta test version, Waters, Milford, MA) as described previously (33). The sample was washed through a 2.1 \(\times\) 30-mm immobilized pepsin column (Porozyme, ABI, Foster City, CA) with 10% glycerol (31, 32) to get a final pH read of 2.5. A 50-\(\mu\)l aliquot of the quenched sample (~200 pmol of protein sample) was then injected on to a chilled nano-UPLC sample manager (beta test version, Waters, Milford, MA) as described previously (33). The sample was washed through a 2.1 \(\times\) 30-mm immobilized pepsin column (Porozyme, ABI, Foster City, CA) with 10% glycerol (31, 32) to get a final pH read of 2.5. A 50-\(\mu\)l aliquot of the quenched sample (~200 pmol of protein sample) was then injected on to a chilled nano-UPLC sample manager (beta test version, Waters, Milford, MA) as described previously (33). The sample was washed through a 2.1 \(\times\) 30-mm immobilized pepsin column (Porozyme, ABI, Foster City, CA) with 10% glycerol (31, 32) to get a final pH read of 2.5. A 50-\(\mu\)l aliquot of the quenched sample (~200 pmol of protein sample) was then injected on to a chilled nano-UPLC sample manager (beta test version, Waters, Milford, MA) as described previously (33). The sample was washed through a 2.1 \(\times\) 30-mm immobilized pepsin column (Porozyme, ABI, Foster City, CA) with 10% glycerol (31, 32) to get a final pH read of 2.5. A 50-\(\mu\)l aliquot of the quenched sample (~200 pmol of protein sample) was then injected on to a chilled nano-UPLC sample manager (beta test version, Waters, Milford, MA) as described previously (33). The sample was washed through a 2.1 \(\times\) 30-mm immobilized pepsin column (Porozyme, ABI, Foster City, CA) with 10% glycerol (31, 32) to get a final pH read of 2.5. A 50-\(\mu\)l aliquot of the quenched sample (~200 pmol of protein sample) was then injected on to a chilled nano-UPLC sample manager (beta test version, Waters, Milford, MA) as described previously (33). The sample was washed through a 2.1 \(\times\) 30-mm immobilized pepsin column (Porozyme, ABI, Foster City, CA) with 10% glycerol (31, 32) to get a final pH read of 2.5. A 50-\(\mu\)l aliquot of the quenched sample (~200 pmol of protein sample) was then injected on to a chilled nano-UPLC sample manager (beta test version, Waters, Milford, MA) as described previously (33). The sample was washed through a 2.1 \(\times\) 30-mm immortalized pepsin column (Porozyme, ABI, Foster City, CA) with 10% glycerol (31, 32) to get a final pH read of 2.5. A 50-\(\mu\)l aliquot of the quenched sample (~200 pmol of protein sample) was then injected on to a chilled nano-UPLC sample manager (beta test version, Waters, Milford, MA) as described previously (33). The sample was washed through a 2.1 \(\times\) 30-mm immortalized pepsin column (Porozyme, ABI, Foster City, CA) with 10% glycerol (31, 32) to get a final pH read of 2.5. A 50-\(\mu\)l aliquot of the quenched sample (~200 pmol of protein sample) was then injected on to a chilled nano-UPLC sample manager (beta test version, Waters, Milford, MA) as described previously (33). The sample was washed through a 2.1 \(\times\) 30-mm immortalized pepsin column (Porozyme, ABI, Foster City, CA) with 10% glycerol (31, 32) to get a final pH read of 2.5. A 50-\(\mu\)l aliquot of the quenched sample (~200 pmol of protein sample) was then injected on to a chilled nano-UPLC sample manager (beta test version, Waters, Milford, MA) as described previously (33).

Sequence identifications were made from MS\(^{E}\) data from undeuterated samples using ProteinLynx Global Server 2.4 (beta test version) (Waters) (37, 38) and searched against the sequence of KATms with no enzyme specified and no modifications of amino acids. Identifications were only considered if they appeared at least twice in three replicate runs. The precur sor ion mass tolerance was set at <10 ppm, and fragment ion tolerance was set at <20 ppm. Only those peptides that satisfied the above criteria through database search pass 1 were selected (37). The default criteria for false positive identification (value = 4) was applied. It should be noted that MS\(^{E}\) does not produce quadrupole-isolated tandem MS (MS/MS) spectra and hence is not optimal for submission to traditional search engines. These identifications were mapped to subsequent deuteration experiments using prototype custom software (HDX...
browser, Waters). Data on each individual peptide at all time points were extracted and exported to HX-Express (39) for analysis. A total of 48 peptide fragments yielded primary sequence coverage of 90% (Table 1).

Continuous instrument calibration was carried out with Glu-fibrinogen peptide at 100 fmol/L. We also visually analyzed the data to ensure that only well resolved peptide isotopic envelopes were subjected to quantitative analysis. A control experiment was carried out to calculate the deuterium back exchange during the experiment by incubating ligand-free PKA regulatory subunit (RI (91–244)) with deuterated buffer for 24 h at room temperature. All reported deuterium exchange values were corrected for a 32.7% back exchange by multiplying the raw centroid values by a multiplication factor of 1.49 (6, 10).

HDXMS results for the cAMP-binding domain of KATms were mapped onto the homology-modeled cAMP-bound structure generated on the basis of the crystal structures of the CNB domains of PKA RI (Protein Data Bank code 1RGS) using SWISS-MODELLER (40). Differences in exchange of 0.5 deuterons were considered significant and mapped onto the structural models.

RESULTS

Conformational Changes in KATms Monitored by BRET—We have recently utilized BRET as a means of monitoring conformational changes in proteins that interact with cyclic nucleotides (31, 41). BRET is based on energy transfer between luminescence donor and fluorescent acceptor proteins (e.g. Renilla luciferase and green fluorescence protein (GFP)) (42) and is dependent on the distance between the donor and the acceptor, their spectral overlap, and their orientation with respect to each other. Because the efficiency of resonance energy transfer depends on the 6th power of the distance between donor and acceptor, even a minimal movement of the two moieties toward each other or away from each other would result in a change in the BRET ratio, which can be monitored in a non-destructive way in solution.

cAMP-mediated regulation of acetyltransferase activity of KATms (29) suggested that conformational changes occur upon cAMP binding to the CNB domain. We therefore attempted to probe these cAMP-dependent conformational changes in KATms using BRET. We used a fusion protein construct of KATms inserted between GFP2 and luciferase (Fig. 1A) (41). The addition of cAMP to lysates expressing the KATms construct led to a significant increase in BRET ratio (Fig. 1B). The concentration of cAMP required to induce this BRET ratio change was similar to the affinity of cAMP binding to purified full-length KATms (Fig. 1C) (29). (S)p-cAMPS was able to induce a BRET ratio increase similar to that seen with cAMP (Fig. 1B). The addition of (R)p-cAMPS increased the BRET ratio to a lower extent than that seen for cAMP or (S)p-cAMPS. This is of relevance in experiments that are detailed below.

Acetyl-CoA is used by the AT domain of KATms as a donor of the acetyl group that is transferred to the e-amino acid side chain of a specific Lys residue in the USP, MSMEG_4207 (29). We therefore monitored BRET in the presence of acetyl-CoA and USP at concentrations that would saturate the enzyme. As shown in Fig. 1B, no change in BRET was seen in the presence of
Cyclic AMP Binding Induces Large Conformational Changes throughout CNB Domain—The change in BRET in the KATms sensor in the presence of cAMP is a result of a conformational change that allowed the coming together of GFP and luciferase or a reorientation of the two domains, such that the resonance energy transfer was more efficient. In order to determine the magnitude of conformational change, localize the regions of KATms that undergo significant alteration in structure upon cAMP binding, and compare dynamics in the cAMP-free and bound states, HDXMS was performed with purified KATms.

Deuterium exchange into rapidly exchanging backbone amides in KATms in the presence and absence of cAMP was carried out by incubating all protein samples in buffer containing D₂O (pH 7.0) at 25 °C, followed by quenching and complete pepsin proteolysis under amide exchange quench conditions as described under “Experimental Procedures.” A total of 45 pepsin fragmentation peptides yielded sequence coverage of ~90% (supplemental Fig. 2). Table 1 summarizes the extent of deuteration for all of the peptides from which quantitative data were obtained. Data are reported as the average number of deuterons exchanged after 10 min for at least two independent experiments in the form of a heat map (Fig. 2A). A butterfly plot (43) was used to provide a protein-wide overview of the relative deuterium exchange for apo- and cAMP-bound states for a short time course (0–10 min; Fig. 2B). It should be noted that this plot displays all individual deuterium-exchanged pepsin digest fragments equally and does not factor into the preferential cleavage of pepsin after specific amino acids or regions of the protein. Nevertheless, it provides a snapshot overview of the exchange across the entire protein and complements information in tabular formats.

Both the CNB and AT domains showed regions of high and moderate deuterium exchange at early time points, but interestingly, decreased exchange upon cAMP binding was observable in the CNB domain alone (Fig. 2). Mass spectra for peptides showing the largest magnitude shifts, along with plots showing deuterium exchange versus time, are shown in Fig. 3. No increases in amide exchange upon cAMP binding were observed, indicating that the decreased exchange reflected a combination of cAMP binding interactions and increased ordering of the CNB domain upon cAMP binding. This appeared to be the basis for the allosteric coupling of cAMP binding to the acetyltransferase domain (Fig. 2 and Table 1).

The region showing the largest magnitude changes in deuterium exchange were peptides spanning residues 56–115 in the N-terminal region of the CNB domain. These spanned the PBC containing invariant Glu and Arg residues, which are critical for anchoring the 2′-OH of the ribose sugar and the phosphodiester bond in the R1α subunit of PKA and other CNB domains (supplemental Fig. 1A). The changes that are observed are similar to and consistent with those reported for the CNB domain of PKA (6) and are expectedly pronounced within the PBC region of the protein. Peptide 77–86, containing conserved Gly-85 and Glu-86, the two residues interacting with the ribose moiety of cAMP, showed decreased exchange (~1 deuteron) compared with the apoprotein (Table 1). These two invariant residues correspond to Gly-199 and Glu-200 in PKA R1α, which mediate hydrogen bonding with the 2′-OH moiety of the ribose group of cAMP. Peptide 90–115, containing conserved Arg-95 and Ala-88 residues that interact with the exocyclic oxygen atoms of the phosphodiester bond, showed decreased exchange (~2 deuterons) in the cAMP-bound state (Table 1). These two residues correspond to the invariant Arg (Arg-209 in PKA R1α) and Ala (Ala-202 in PKA R1α) (6). Regions outside the PBC within the CNB domain that showed decreased amide exchange span peptide 56–76 (~3.0-deuteron decrease in exchange) in the cAMP-bound state. An overlapping peptide, 68–76, also showed decreased exchange in the cAMP-bound state. Subtractive analysis enabled localization of cAMP-dependent shifts in deuterium exchange to residues 57–68 (Table 1). Interestingly, this segment contains a conserved Val that has been postulated to contribute to a “hydrophobic sandwich,” which positions the adenosine ring of cAMP. Peptides 41–62, 60–88, and 90–115 containing conserved hydrophobic residues Leu-44, Ile-45, Gly-48, Ile-87, and Ala-97 all showed decreased exchange in the cAMP-bound state (Fig. 2 and Table 1). A peptide, 18–44, from the N terminus also showed decreased exchange in the cAMP-bound state.

HDXMS results for the cAMP-binding domain of KATms were mapped onto the homology-modeled cAMP-bound structure of the CNB domains of PKA R1α (Protein Data Bank code 1RGS) using SWISS-MODELLER (40). Comparison of the cAMP-dependent shifts in relative deuterium exchange across PKA R1α and KATms revealed interesting differences (Fig. 4). In PKA R1α, the regions showing the larger relative shifts in deuterium exchange were localized to the PBC, compared with the rest of the CNB domain (6). In KATms, in contrast, the butterfly plot (Fig. 2) demonstrates that the rest of the CNB domain, outside the PBC, showed larger magnitude shifts in deuterium exchange upon interaction with cAMP. Interestingly, no changes were observable in regions previously reported to be associated with allosteric relays in PKA or Epac (13, 34). This suggests that the CNB domain in KATms functions as a simpler cyclic nucleotide binding-induced switch.

We refer to the region between the N-terminal CNB domain and the C-terminal AT domain as the linker region. In addition to the changes observed at the CNB domain, binding of cAMP induced significant changes in this linker region. For example,
peptide 153–172 showed decreased exchange upon cAMP binding (−1 deuteron), which is also seen with overlapping peptides 153–173, 153–164, and 154–173 (Table 1). Subtractive analysis enabled localization of cAMP-dependent shifts in deuterium exchange to residues 153–164 (Table 1), indicating that effects of cAMP binding to the CNB domain were transmitted to distal regions of the protein.

Finally, no significant changes in exchange were observed in any peptide in the AT domain upon cAMP binding. We concluded, therefore, that increases in AT activity seen upon cAMP binding to KATms were a consequence of the relay of information transmitted from the CNB domain to the AT domain via the linker region. This might relieve some inhibition of the AT domain present in the non-cAMP-bound conformation of the protein.

**Table 1**

| Peptide no. | Sequence (m/z) (charge) | Position nos. | Maximum exchangeable amides |
|-------------|------------------------|---------------|----------------------------|
|             |                        |               | KATms, cAMP                  |
| 1           | LTEVRAADL (987.54) (+1) | 41–22         | 5.7 ± 0.1                    |
| 2           | FTGCRPSALPEQRLPKAEQPGVQVL (723.90) (+4) | 18–41         | 5.6 ± 0.1                    |
| 3           | QRLPKAEQPGVQVL (724.934) (+2) | 32–44         | 5.6 ± 0.1                    |
| 4           | QGQLVRQGDPMFLIESGRV (800.76) (+3) | 41–62         | 5.7 ± 0.1                    |
| 5           | IQRGDPALTME (624.83) (+1) | 45–55         | 4.2 ± 0.0                    |
| 6           | LIEGSQGSHVADGPVPVL (1079.11) (+2) | 56–76         | 4.2 ± 0.0                    |
| 7           | VADGPVPVL (880.510) (+1) | 68–76         | 4.0 ± 0.0                    |
| 8           | [IESGRQVSH]- [57–68] (+1) | 77–86         | 4.0 ± 0.0                    |
| 9           | LRDAPRTATVQAAEPIVGVWGDRADF (928.164) (+3) | 70–105        | 4.0 ± 0.0                    |
| 10          | EPIVGVWGDRADF (1460.73) (+1) | 103–115       | 4.0 ± 0.0                    |
| 11          | PVGIVGVWGDRADF (666.341) (+2) | 104–115       | 4.0 ± 0.0                    |
| 12          | DTIHLQPMAGM (1143.58) (+1) | 91–102        | 4.0 ± 0.0                    |
| 13          | ITIPVQRTGW (748.42) (+2) | 140–152       | 4.0 ± 0.0                    |
| 14          | FYLRPVLGDPVTLNPGVE (757.74) (+3) | 153–172       | 4.0 ± 0.0                    |
| 15          | FYLRPVLGDPVTLNPGVE (806.766) (+3) | 153–172       | 4.0 ± 0.0                    |
| 16          | YLRPVLGDPVTLNPGVE (757.746) (+3) | 153–172       | 4.0 ± 0.0                    |
| 17          | ERTLNGPVF (1161.581) (+1) | 164–173       | 4.0 ± 0.0                    |
| 18          | [LRPVLQPGD] (155–164) (+1) | 195–200       | 4.0 ± 0.0                    |
| 19          | LLYFED (785.368) (+1) | 197–207       | 4.0 ± 0.0                    |
| 20          | FEVDAYDH (995.404) (+1) | 207–218       | 4.0 ± 0.0                    |
| 21          | FEVDAYDHFWV (714.32) (+2) | 207–218       | 4.0 ± 0.0                    |
| 22          | VVWMTEGALGPVIADARF (866.506) (+2) | 207–223       | 4.0 ± 0.0                    |
| 23          | VMTEGALGPVIADARF (1272.65) (+1) | 208–220       | 4.0 ± 0.0                    |
| 24          | TEGALGPVIADARF (1042.54) (+1) | 220–232       | 4.0 ± 0.0                    |
| 25          | TEGALGPVIADARF (708.882) (+2) | 210–222       | 4.0 ± 0.0                    |
| 26          | RFVREGHATMAE (759.37) (+2) | 222–234       | 4.0 ± 0.0                    |
| 27          | RFVREGHATMAE (1214.55) (+2) | 224–234       | 4.0 ± 0.0                    |
| 28          | VAFTVGDG (823.381) (+1) | 235–247       | 4.0 ± 0.0                    |
| 29          | VAFTVGDG (894.933) (+2) | 235–251       | 4.0 ± 0.0                    |
| 30          | VAFTVGDG (894.933) (+2) | 235–252       | 4.0 ± 0.0                    |
| 31          | VAFTVGDG (894.933) (+2) | 235–252       | 4.0 ± 0.0                    |
| 32          | VAFTVGDG (894.933) (+2) | 235–252       | 4.0 ± 0.0                    |
| 33          | LIVSN (616.363) (+1) | 256–261       | 4.0 ± 0.0                    |
| 34          | LIVSN (616.363) (+1) | 256–261       | 4.0 ± 0.0                    |
| 35          | LIVSN (616.363) (+1) | 256–261       | 4.0 ± 0.0                    |

Deuterons exchanged

- Average number of deuterons exchanged determined from centroid analysis of isotopic envelopes of pentide digest fragments of KATms following a 10-min exposure to deuterium oxide. Values reported are the mean and S.D. from at least two independent experiments.

Differential Effects of cAMP Analogs 8-Bromo-(S)\(_p\)-cAMPS and 8-Bromo-(R)\(_p\)-cAMPS—We have shown earlier that the CNB domain in KATms binds (S)\(_p\)-cAMPS with an affinity comparable with that of cAMP (IC\(_{50} = 65 \pm 12.3\) nM), whereas the corresponding (R)\(_p\) isomer showed a much lower affinity of interaction (IC\(_{50} = 231.1 \pm 1.6\) µM) (29). Not only does this underscore the significance of the equatorial oxygen atom in the exocyclic oxygens of the cyclic phosphodiester bond of cAMP for binding tightly at the PBC of CNBs (1, 6); this also highlights the importance of equatorial oxygen-guanidinium moiety interactions with Arg-95 in anchoring cAMP to the pocket.

It was of interest to test the effects of these analogs on allosteric, as has been described earlier for the regulatory subunit of
PKA (10, 44). We have used 8-bromo-substituted cAMP analogs in the current study because they are more stable in solution (45). ($S_p$)-Bound KATms showed changes in deuterium exchange nearly identical to those seen with cAMP, with peptide 56–76 alone showing slightly higher exchange (~1 deuterium) in the complex with ($S_p$)-cAMPS, than in the complex with cAMP (Table 1 and Figs. 3 and 5). Factoring in the lower affinity of ($S_p$)-cAMPS for KATms compared with ($S_p$)-cAMPS.
(29), we carried out HDXMS studies under conditions where a majority of protein would be bound to \((R_p)\)-cAMPS. Interestingly, there was no difference in deuterium exchange between the apoprotein and protein bound to \((R_p)\)-cAMPS (Fig. 5). This was surprising because in comparative studies with PKA, the PBC pocket did show decreased exchange in the presence of \((R_p)\)-cAMPS, although the binding affinity of \((R_p)\)-cAMPS was lower (6). Therefore, our results seem to indicate a high level of dynamics at the binding site of KATms in the presence of \((R_p)\)-cAMPS that was faster than the intrinsic rate of deuterium exchange (8).

**Linker Region Is Important for Propagating cAMP-induced Conformational Changes in KATms**—HDXMS suggested that the effects of cAMP did not result in a dramatic conformational change in the AT domain. Biochemical evidence for this was provided by performing acetyltransferase assays with KATms in the presence and absence of cAMP. As shown in Fig. 6A, although the \(K_m\) for acetyl-CoA was not altered in the presence of cAMP, the reaction rate was enhanced in the presence of cAMP (Fig. 6B). This suggested that the linker region is important for propagating cAMP-induced conformational changes in KATms.

**Figures**

**FIGURE 3.** Mass spectra of representative peptides analyzed in HDXMS. A, electrospray ionization quadrupole TOF mass spectra for three pepsin digest fragments of KATms, showing significant differences in deuterium exchange upon cAMP/(S_p)-cAMPS binding. Comparison in the apo and ligand-bound states is shown following 10-min deuterium exchange. i, isotopic envelope for peptide in apo-KATms; ii, isotopic envelope for peptide in \((R_p)\)-cAMPS-bound KATms; iii, isotopic envelope for peptide in \((S_p)\)-cAMPS-bound KATms; iv, isotopic envelope in cAMP-bound KATms; v, undeuterated sample. Mass spectra for pepsin fragment peptides shown are as follows: KATms(90–115), \(m/z = 928.164, z = 3\); KATms(56–76), \(m/z = 1079.11, z = 2\); KATms(153–173), \(m/z = 757.74, z = 3\). B, kinetic plots of deuterium exchange for the three peptides above: apo-KATms (open circle), cAMP-bound (green); \((S_p)\)-cAMPS-bound (red), and \((R_p)\)-cAMPS-bound (blue).

**FIGURE 4.** Conformational changes in the CNB domain of PKA and KATms. A, structure of cAMP-bound CNB-A of PKA R-subunit (Protein Data Bank code 1RGS) (58), highlighting in blue the regions showing decreased deuterium exchange in the presence of cAMP. Residues Glu-200 and Arg-209 coordinate binding to the ribose 2\(^{-}\)-OH, and the equatorial and axial oxygen atoms of cAMP are displayed as sticks. Arrow A highlights the Arg-209-equatorial oxygen-Asp-170 allosteric relay in PKA R1. Arrow B highlights the hydrophobic switch mediated by Leu-203 and Ile-204 with \(\alpha\)-B/C helices. B, CNB domain of KATms was modeled in the SWISS MODEL automated server using structural coordinates of PKA R1 (Protein Data Bank code 1RGS) as a template. cAMP-bound KATms CNB domain model highlighting in blue the regions showing decreased deuterium exchange in the presence of cAMP.
FIGURE 5. HDXMS of KATms in the presence of \((S_p)-cAMPS\) or \((R_p)-cAMPS\). A, butterfly plot showing the average relative fractional exchange (deuterons exchanged/maximum exchangeable amides) \((y\) axis) for all of the pepsin digest fragments of KATms listed from N to C terminus \((x\) axis) for \((R_p)-cAMPS\)-bound (top) and \((S_p)-cAMPS\)-bound (bottom); each trace represents a time point for deuterium exchange: 1 min (orange), 2 min (brown), 5 min (blue), and 10 min (black). These data represent the results from two independent experiments. B, difference plot localizing changes in deuterium exchange between cAMP analogs \((R_p)-cAMPS\) and \((S_p)-cAMPS\). Color schemes for the plots are the same as in A. Two blue boxes with dashed lines highlight the regions of the protein showing decreased exchange in the presence of \((S_p)-cAMPS\) binding. Domain organization of KATms (N-terminal, CNB, and C-terminal acetyltransferase domains connected by a linker region) is shown as an inset.

FIGURE 6. Allostery in KATms mediated via the linker region. A, KATms was incubated with varying concentrations of acetyl CoA, either in the presence or absence of cAMP. Western blot analysis of the samples was performed using acetyl lysine antibody, and densitometric analysis of immunoreactive bands was analyzed as detailed under “Experimental Procedures.” The data shown represent the mean ± S.E. of assays performed three times. B, varying concentrations of USP were used in an acetylation reaction using KATms either in the presence or absence of cAMP. Samples were subjected to Western blot analysis using an acetyl lysine antibody. The blot shown is representative of assays performed three times and demonstrates that in the presence of cAMP, a lower concentration of USP can be acetylated more efficiently. C, a construct of the CNB and the linker domain fused at the N terminus to GFP and the C terminus to luciferase (inset) was transfected into HEK293T cells. Lysates were prepared, and BRET was measured in the presence of varying concentrations of cAMP. Values represent the mean ± S.E. (error bars) of assays repeated in duplicate at least three times.
of cAMP, the $V_{max}$ of acetylation of its substrate USP was increased. This indicated enhancement of catalysis in the presence of cAMP without a dramatic change in binding affinities to acetyl-CoA. Acetylation of USP was monitored in the presence and absence of cAMP, and as shown in Fig. 6B, the presence of cAMP increased the efficiency of acetylation of low concentrations of USP. This suggests that the conformational change induced by cAMP facilitates more efficient binding of USP to the catalytic site.

HDXMS indicated significant increases in exchange in the linker region in the presence of cAMP. To confirm that the linker region was important in propagating the effects of cAMP binding to the acetyltransferase domain, we generated an additional construct for BRET analysis that was truncated before the AT domain, encompassing the CNB domain and the linker region. As shown in Fig. 6C, an increase in the BRET ratio was observed in the presence of cAMP, at concentrations that were close to that seen for the full-length protein (Fig. 1C). Therefore, these results show that the linker region does indeed participate in the relay of information from the CNB domain to the AT domain in KATms, as suggested by the HDXMS data, emphasizing the complete convergence and consistency between BRET assays and HDXMS.

**Mutations in Linker Region Abolish cAMP-mediated Activation of AT Activity**—A PXXP motif (residues 157–160) is found in the peptide in the linker region, which showed maximum changes in response to cAMP in HDXMS analysis (residues 153–164). To establish that cAMP binding to the CNB domain is allosterically coupled to the PXXP motif in the linker domain, we mutated residues Pro-157 and Pro-160 to Ala. Both proline residues were mutated to completely disrupt the structure of the linker region. The purified mutant protein showed a similar affinity for cAMP as the wild type protein (Fig. 7A). We monitored the AT activity of the wild type and mutant proteins in the presence of cAMP, $(S_p)$-cAMPS, or $(R_p)$-cAMPS using Western blot analysis and a coupled acetyltransferase enzyme assay (supplemental Fig. 3). As shown in Fig. 7, B and C, $(S_p)$-cAMP was able to enhance acetylation of USP by wild type KATms to a similar extent as seen with cAMP. This is in agreement with the BRET ratio and HDXMS data that demonstrated similar conformational changes induced by $(S_p)$-cAMPS and cAMP (Figs. 1B and 3). The presence of $(R_p)$-cAMPS did not result in an increase in the rate of acetylation of USP, which can be correlated with the lower change in BRET that was induced in the KATms sensor in the presence of $(R_p)$-cAMPS (Fig. 1B). Importantly, no increase in the rate of acetylation of USP in the presence of cAMP was observed in the mutant protein, indicating the essential requirement of the proline residues in the relay of conformational changes from the CNB domain to the AT domain (Fig. 7, B and C, and supplemental Fig. 3). Activation of AT activity by $(S_p)$-cAMPS was also lost in the mutant protein, indicating that similar conformational changes are induced by $(S_p)$-cAMPS and cAMP, in agreement with the HDXMS data (Table 1).

The PXXP motif within the linker region suggested that either (a) it was responsible for inhibition of the KATms catalytic domain via intramolecular interactions, as has been shown in protein kinase C (46), or (b) it facilitated interactions with substrate in an intermolecular manner upon the addition of cAMP, or (c) a combination of both effects. Our BRET results
suggested that cAMP binding to the CNB domain was coupled to repositioning of the PXXP motif leading to enhancement of catalysis.

**Linker-mediated Conformational Changes in Presence of cAMP Are Conserved in Rv0998**—We have earlier shown that the ortholog of KATms from *M. tuberculosis* (product of the Rv0998 gene; KATmt) was able to acetylate USP in a strictly cAMP-dependent manner, and the affinity of KATmt for cAMP (IC₅₀ = 100 μM) was lower than that of KATms (29). A sequence alignment of these two proteins shows striking conservation of residues in the linker region, which showed the maximum shifts in deuterium exchange upon cAMP binding (residues 158–165; Fig. 8A). Therefore, Pro residues at 160 and 163 were mutated to Ala in KATmt, and the purified protein was interacted with [³H]cAMP in the presence and absence of varying concentrations of unlabeled cAMP. Radioactivity bound to the protein was monitored following filtration through nitrocellulose filters, and data obtained were analyzed by GraphPad Prism. Data shown represent the mean ± S.E. (error bars) of triplicate experiments. C, either wild type or mutant KATmt was incubated with USP and acetyl-CoA, in the presence or absence of cAMP. Samples were then subjected to Western blotting with the acetyl lysine antibody. Following blotting, the gel was stained with Coomassie to detect USP.

**FIGURE 8. Conserved mechanism of allosteric activation of KATmt by cAMP.** A, sequence alignment of Rv0998 (KATmt) and MSMEG_5458 (KATms). Shown in green are residues in the CNB domain. Residues highlighted in yellow indicate regions that showed maximum differences in HDXMS in the presence of cAMP, which are found in the linker region. Residues highlighted in red represent the acetyltransferase domain. The arrowheads point to the conserved proline residues that were mutated in KATms and KATmt in this study. B, Pro residues at 160 and 163 were mutated to Ala in KATmt, and the purified protein was interacted with [³H]cAMP in the presence and absence of varying concentrations of unlabeled cAMP. Radioactivity bound to the protein was monitored following filtration through nitrocellulose filters, and data obtained were analyzed by GraphPad Prism. Data shown represent the mean ± S.E. (error bars) of triplicate experiments. C, either wild type or mutant KATmt was incubated with USP and acetyl-CoA, in the presence or absence of cAMP. Samples were then subjected to Western blotting with the acetyl lysine antibody. Following blotting, the gel was stained with Coomassie to detect USP.

**DISCUSSION**

In this current study, the unique combination of BRET assays and HDXMS has enabled us to identify and measure the conformational dynamics of cAMP-dependent activation of a novel cAMP-binding protein. We have earlier shown that not only is BRET a useful tool to monitor cyclic nucleotide-dependent conformational changes in an isolated GAF domain (41), but it can also report on allostery in the full-length cGMP-binding, cGMP-specific phosphodiesterase, PDE5 (31). These results formed the basis for our attempt to monitor conformational changes using BRET in full-length KATms, where biochemical evidence indicated that cAMP binding regulated the dynamics of cAMP-induced conformational change in KATms and KATmt are similar and involve identical residues in the linker region. Therefore, despite the early divergence of mycobacteria into fast growing (e.g. *M. smegmatis*) and slow growing (*M. tuberculosis*) species, the mechanism by which cAMP activates an associated acetyltransferase domain has been conserved.
associated catalytic domain that possessed acetyltransferase activity (29, 30).

There are three possible modes of how cAMP might enhance acetyltransferase activity in KATms. These include (a) relief of inhibition by the CNB domain upon binding to cAMP, (b) facilitation of substrate/cofactor binding to the enzyme catalytic site, and (c) rearrangements of residues at the acetyltransferase catalytic core, leading to enhancement of catalysis. Mechanisms a and b, however, may in part coincide, as is seen in Epac, where the CNB occludes the active site by steric hindrance (13). Comparison of Michaelis-Menten kinetic parameters in the cAMP-free and -bound states shows no difference in the $K_{on}$ for acetyl-CoA (Fig. 6A), indicating that the enhanced acetyltransferase activity is the result of an increase in the catalytic rate constant ($k_{cat}$). In contrast, cAMP binding enhances the affinity of the enzyme for USP (Fig. 6B), suggesting that activation is at least partially through mode c.

HDXMS results show no differences in deuterium exchange between the cAMP-bound and free states for any part of the AT domain, thereby precluding a model for enhanced catalysis resulting from a large scale rearrangement of active site residues (mode c). Together, these results suggest that the basis for activation is via a relief of inhibitory effects of the CNB domain on AT activity by cAMP, resulting in enhancement of substrate interactions.

Binding of cAMP to KATms resulted in decreases in deuterium exchange in the CNB domain and linker alone. This points to increased ordering of the CNB domain and, consequently, reduced dynamics upon cAMP binding. The regions showing decreased exchange are consistent with other CNB domain-containing proteins, such as the PKA regulatory subunit and Epac (9, 11, 13). Interestingly, however, the relative decreases in exchange across CNB domains in KATms differ from that seen in the PKA R-subunit, for the same time course of deuterium exchange assessed. The reduction in exchange at the PBC in KATms is larger than in the other parts of the CNB domain, especially those regions mediating interactions with the adenine ring. In contrast, in KATms, the magnitude shifts in deuterium exchange were larger in the regions spanning the adenine interaction regions relative to the PBC. This is significant because it underscores the differential importance of functional moieties of cAMP in the mycobacterial and other CNB domains. In PKA and Epac, HDXMS suggests that contacts within the PBC are more critical for cAMP binding (9, 11, 13), whereas in KATms, our results show that hydrophobic contacts with the adenine are just as important as, if not more important than, contacts at the PBC.

In this regard, KATms (which is a dimer in solution) (29) shows interesting parallels with CAP from E. coli. In the CAP dimer, a long C/C' helix connects the CNB domain with the DNA binding domain. The C/C' helices of each monomer are solely responsible for maintaining the dimeric interface (47). cAMP binding has been proposed to activate CAP through coiled helix transitions in the C/C' helical region. These transitions have been proposed to be mediated by stabilization of CAP by contacts with the adenine ring, by interactions with the ribose and phosphodiester bonds, or through rearrangements of the aromatic cap residue (47). Given the larger magnitude shifts in regions of the protein that interact with the adenine ring, stabilization of KATms via interactions with the adenine ring might result in increased coil to helix transitions in the interdomain linker region, resulting in rearrangement of the AT domain and enhanced catalysis. Our BRET results on the truncated CNB-linker domain construct are further consistent with such a model. We were unable to monitor effects of substrate on cAMP-dependent conformational changes in KATms by HDXMS because no competitive inhibitors of KATms have as yet been identified.

HDXMS has been a valuable method for discerning the mechanistic aspects of allostery in cAMP-binding domains. In the case of Epac2, allostery upon cAMP binding is propagated via a conserved hydrophobic aromatic switch residue, Phe-435 (hinge region) and hydrophobic Leu-408 in the PBC. In PKA RIo, the corresponding residues include Leu-203, Ile-204 (PBC), and Tyr-229 (hinge) (9). Although the importance of these residues in the relay has been demonstrated in Epac2, they have not been shown directly to be critical in allosytic activation of PKA (10). In PKA, allosytic communication relays are propagated from the PBC binding site via a conserved Asp-170, which couples the PBC and the intersubunit interface with the catalytic subunit (10, 15, 44, 48). All of these studies identify two distinct steps in cAMP action. Binding of cAMP to the PBC is followed by allosytic relays that rely on the specific interactions of the cyclic phosphodiester bond. Significantly, no changes in amide exchange were seen in any regions of KATms outside of the PBC that flanks the exocyclic equatorial oxygen of cAMP. This was surprising because in both Epac and PKA, such changes have provided insights into allosytic relays leading from this signaling locus. Importantly, there is no conservation of a residue equivalent to Asp-170 in either Epac or KATms, indicating that its role is unique to allosytic activation in PKA.

Mutation of Arg-95 reduced cAMP binding to KATms, underscoring the importance of this residue for anchoring cAMP interactions with the CNB domain (29), as is seen with all CNB domains. In agreement with this, no significant change in BRET was seen in the presence of cAMP with the R95K mutant protein (Fig. 1D), validating the use of BRET to monitor cAMP-dependent conformational changes in KATms. Unlike in Epac or PKA, HDXMS with KATms revealed that there is no conserved allosteric network radiating from the cyclic phosphate, via the conserved Arg-95 in the PBC, to the AT catalytic core. It is therefore likely that KATms is an example of a primordial CNB domain where conformational changes are a consequence of binding-induced ordering alone.

In the CNB domains of PKA and Epac, parallel intricate allosytic relays have evolved for better specificity. Recent studies point to evidence for both conformational selection (6) and induced fit (10) in both PKA and combinations of the two mechanisms in Epac (49, 50), where allosytic relays are critical. The phosphate group is all important in allossty. However, KATms is an example where allostery appears to be linked solely to binding of the adenine base. The exocyclic equatorial oxygen that is so critical in PKA and Epac does not appear to be important for anything other than providing high affinity binding in KATms.
(S_p)-cAMPS and cAMP bind with similar affinities to KATms and markedly enhance acetyltransferase activity (Fig. 7) (29). (R_p)-cAMPS acts as a weak agonist (Figs. 1B and 7C), in contrast to its antagonistic action in Epac (51). In PKA, whereas (S_p)-cAMPS is an agonist (6, 52), (R_p)-cAMPS functions as an antagonist in the PKA holoenzyme, when MgADP is bound to the C-subunit (10, 52). However, if the holoenzyme is incubated with MgADP and substrate peptide, (R_p)-cAMPS functions as an agonist. Further, (R_p)-cAMPS also switches to functioning as a weak agonist in a mutant of the R-subunit where the conserved arginine is replaced by a lysine residue (52).

In KATms, (R_p)-cAMPS shows a 1000-fold decreased binding affinity in comparison with cAMP or (S_p)-cAMPS (29), reflecting the importance of the exocyclic equatorial oxygen atoms for high affinity binding to the PBC of KATms. Comparison of HDXMS of KATms with the (R_p)-cAMPS and (S_p)-cAMPS analogs reveals important differences. Changes seen with (S_p)-cAMPS were very similar to those seen in cAMP-bound KATms. In contrast, no changes in deuterium exchange were observable upon binding of (R_p)-cAMPS, although binding of (R_p)-cAMPS allowed a modest increase in acetyltransferase activity (Fig. 1B and supplemental Fig. 3). Because the observed rate of HD exchange, \( k_{obs} \), is dependent on both off-on kinetic rates of complexation and the intrinsic deuterium exchange rate (53), our results suggest that the kinetic off-rate for (R_p)-cAMPS is probably much faster (and/or the rate of reassociation is much slower) than the observed rate of deuterium exchange. This, therefore, would preclude capturing the binding event in (R_p)-cAMPS and binding-induced allosteroy in KATms HDXMS.

The only region outside the putative CNB domain that showed any differences in deuterium exchange was the linker region (residues 153–168). That these changes in the linker region were important was also inferred from our BRET analyses because the construct containing the CNB domain with the linker region showed the same magnitude of BRET responses upon cAMP binding as full-length KATms (Fig. 6C). This altered dynamics of the linker region upon cAMP binding suggests that the relief of inhibition by the N-terminal CNB domain on the acetyltransferase domain is achieved via the linker region, and this was supported by mutational analysis in the linker region (Figs. 7 and 8). In Epac, the linker region, which is entirely \( \alpha \)-helical and leads away from the core of the CNB domain, is an important lid region for capping interactions of cAMP at the PBC (13). An analogous role for the linker in KATms seems less plausible because the putative linker is very long (~25 residues), and furthermore, the decreased exchange is only observed at the C-terminal end of the linker region, away from the CNB domain. However, secondary structure predictions do indicate that the residues between the CNB domain and the acetyltransferase domain can form a helix (supplemental Fig. 4) (54). Moreover, it is important to note that the mechanisms of activation via the linker regions are conserved in KATms and KATm (Figs. 7 and 8), despite dramatic changes in the affinity of cAMP binding. Our results also fit well with the emerging role of the linkers as integral parts of allosteric networks (55), a phenomenon we have observed earlier in receptor guanylyl cyclases (56).

In conclusion, therefore, HDXMS reveals a mechanism for cAMP-mediated activation of KATms explainable either through induced fit or conformational selection models or a combination of both models (Figs. 7 and 8), as we have reported earlier for PKA (6, 9). According to the induced fit model, reduction in dynamics upon binding of cAMP to the CNB domain is propagated to the linker region connecting the CNB and AT domains, resulting in activation via rearrangement of domains and relief of inhibition. In the conformational selection model, cAMP binds and shifts the equilibrium toward the active cAMP-bound state. Here the linker region assumes a different conformation in the active cAMP-bound state, promoting enhanced catalysis through relief of inhibition. In the induced fit model, cAMP binding drives the conformational changes, whereas in the conformational selection model, cAMP binding shifts the equilibrium to favor the active state. Nevertheless, in both models, activation is achieved solely through stabilization of the linker region and the PBC following binding by cAMP, obviating the need for intricate parallel allosteric relays for cAMP action. This is in contrast to what has been seen with eukaryotic CNBs that have conserved high affinity binding sites coupled to allosteric relays (57).

Our HDXMS results are highly consistent with BRET analysis, providing powerful insights into the mechanism of cAMP action. This powerful convergence of results from two independent techniques reaffirms the complementary power of solution biophysical tools in unraveling the mechanistic basis of regulation of proteins in the absence of high resolution structural information.

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