A Model for Agonism and Antagonism in an Ancient and Ubiquitous cAMP-binding Domain*1

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The cAMP-binding domain (CBD)2 represents a conserved regulatory motif that allosterically modulates the function of a group of diverse proteins, thereby translating the cAMP signal into a controlled biological response. The main receptor for cAMP in mammals is the ubiquitous regulatory (R) subunit of protein kinase A. Despite the recognized significant potential for pharmacological applications of CBDSs, only one group of competitive inhibitor antagonists is known: the (Rp)-cAMPS family of phosphorothioate cAMP analogs, in which the equatorial exocyclic oxygen of cAMP is replaced by sulfur. It is also known that the diasteroisomer (S)p-cAMPS with opposite phosphorous chirality is a cAMP agonist, but the molecular mechanism of action of these analogs is currently not fully understood. Previous crystallographic and unfolding investigations point to the enhanced CBD dynamics as a key determinant of antagonism. Here, we investigate the (Rp)- and (S)p-cAMPS-bound states of R(CBD-A) using a comparative NMR approach that reveals a clear chemical shift and dynamic NMR signature, differentiating the (S)p-cAMPS agonist from the (Rp)-cAMPS antagonist. Based on these data, we have proposed a model for the (Rp/S)p-cAMPS antagonism and agonism in terms of steric and electronic effects on two main allosteric relay sites, Ile163 and Asp170, respectively, affecting the stability of a tertiary inhibitory complex formed by the effector ligand, the regulatory and the catalytic subunits of protein kinase A. The proposed model not only rationalizes the existing data on the phosphorothioate analogs, but it will also facilitate the design of novel cAMP antagonists and agonists.

The cAMP-binding domain (CBD)3 represents a conserved regulatory motif that modulates the function of a diverse group of proteins, including protein kinase A (PKA) in eukaryotes (1), transcription factors in bacteria (catabolite activator protein (2–11), guanine exchange factors (EPAC) (12–15), and ion channel proteins (both hyperpolarization-activated cyclic nucleotide-dependent channels and cyclic nucleotide-gated channels) (16, 17). All CBDS share a conserved architecture with a contiguous β-subdomain nested within a noncontiguous α-subdomain (18, 19). The β-subdomain consists of an eight-stranded β-barrel, where the cAMP phosphate-binding cassette (PBC) is located. Upon cAMP binding, its signal is propagated through the α/β-subdomain interface, causing a rearrangement of the orientation of the three α-helices in the α-subdomain. It is this α-subdomain rearrangement that ultimately controls the activity of the functional unit coupled to cAMP (20).

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

The crystal structures of the regulatory subunit of PKA (R) bound to both phosphorothioate analogs have been solved at 2.3 Å resolution and are overall very similar to each other (i.e. Cα root mean square deviation of 0.34 Å) and to the cAMP-bound state (i.e. Cα root mean square deviation of 0.5 Å) as well (27). Despite the overall similarity of these three structures, subtle local conformational differences were found for the conserved arginines in the PBC (i.e. Arg209 for CBD-A and Arg333 for CBD-B). For example, the intermolecular distances indicate that the phosph(oxy)ate-Arg209 guanidinium interaction is significantly tighter for (Rp)-cAMPS as compared with (S)p-cAMPS and cAMP, although the affinity of the (Rp)-surrogate for this CBD is lower than that of the (S)p-analog and of cAMP (27). In addition, the crystallographic investigation revealed...
that the \((R_p)\)-cAMPS bound state is probably more dynamic than the \((S_p)\)-cAMPS- and cAMP-bound forms, based on its higher B-factors (27). It has also been shown that the Gibbs free energy of urea unfolding of \((R_p)\)-cAMPS-bound R is similar to that of cAMP-free R, whereas the thermodynamics of unfolding for \((S_p)\)-cAMPS compares well with that of the more thermodynamically stable cAMP-bound R (28).

Here, we further investigate by NMR the effects of \((R_p)\)- and \((S_p)\)-cAMPS binding. For this purpose, we focus on the 119–244 Rlaα fragment, which maps to CBD-A of the R-subunit of PKA and represents its minimal central controlling unit (29). Using a comparative NMR strategy based on \(N_z\) exchange spectroscopy to assign different bound states, it was possible to unveil how the internal signaling pathways of CBD-A are differentially perturbed by \((R_p)\)-cAMPS as compared with \((S_p)\)-cAMPS and cAMP. In addition, \(^{15}\)N relaxation measurements (i.e. \(T_{1S}\), \(HN\) NOE, and relaxation-compensated constant time CPMG NMR dispersion) combined with hydrodynamic simulations provide further insight on the sites and time scales of the enhanced dynamics caused by \((R_p)\)-cAMPS. The interpretation of these results in the context of the allosteric networks of CBD-A and of the stereo-electronic effects caused by the oxygen-to-sulfur isolobal substitution has led to the proposition of a molecular model for the antagonism and agonism of the \((R_p)\)- and \((S_p)\)-cAMPS analogs, respectively.

**EXPERIMENTAL PROCEDURES**

**NMR Sample Preparation of Rlaα-(119–244) Bound to Different Ligands**—Samples of uniformly \(^{15}\)N-labeled cAMP-bound Rlaα-(119–244) in MES buffer (50 mM MES, pH 6.5, 100 mM NaCl, and 0.02% NaN\(_3\)) were prepared as previously discussed (29, 30). The Rlaα-(119–244) samples with substoichiometric amounts of cAMP used for the \(N_z\) exchange experiments were obtained through a protocol of unfolding, partial dialyzing out of cAMP and refolding. Specifically, after adding 8 M urea to cAMP-bound Rlaα-(119–244), the sample was dialyzed for 18 h in the presence of 0.5 liters of MES buffer with 6 M urea and 1 mM dithiothreitol. After changing the dialysis buffer three times at regular intervals, the protein was then partially refolded by stepwise dilution of the dialysis solution to 0.5 M urea through the addition of MES buffer with 1 mM dithiothreitol. Essentially complete removal of urea was obtained by a final dialysis against the MES buffer, which resulted in a sample with NMR-detectable amounts of both cAMP-bound and free states of Rlaα-(119–244) at a total concentration of \(-0.1\) mM. The addition of 50 \(\mu\)M \((S_p)\)-cAMPS phosphorothioate cAMP analog (Sigma) to this sample resulted in another NMR sample with NMR-observable amounts of both cAMP-bound and \((S_p)\)-cAMPS-bound Rlaα-(119–244). This sample was used to assign the \((S_p)\)-cAMPS-bound Rlaα-(119–244) through \(N_z\) exchange spectroscopy. The sample with both cAMP-bound and \((R_p)\)-cAMPS-bound (Sigma) Rlaα-(119–244) was prepared similarly. Samples containing only Rlaα-(119–244) bound to the \((S_p)\)-cAMPS or \((R_p)\)-cAMPS analogs were prepared by extensively rather than partially dialyzing the protein under denaturing conditions and then refolding. Specifically, we added urea to 6 ml of 0.15 mM Rlaα-(119–244) to a final urea concentration of 8 M. After keeping the resulting solution at room temperature for 30 min, it was dialyzed against 8 M urea buffer (50 mM MES, pH 6.5, 100 mM NaCl, and 1 mM dithiothreitol) for 16 h with three buffer changes. The protein solution was then dialyzed extensively with 6 M urea for 24 h to completely remove cAMP. After refolding the protein as indicated above, 1 mM excess \((S_p)\)-cAMPS analog was added. The \((R_p)\)-cAMPS-bound Rlaα-(119–244) sample was prepared likewise.

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

**\(N_z\) Exchange**—The assignment of the backbone amides of Rlaα-(119–244) bound to \((S_p)\)-cAMPS was obtained from that of cAMP-bound Rlaα-(119–244) through \(N_z\) exchange spectra (36–38) acquired for a sample with NMR-observable amounts of both cAMP-bound and \((S_p)\)-cAMPS-bound Rlaα-(119–244). Similarly, the backbone amides of Rlaα-(119–244) bound to \((R_p)\)-cAMPS were assigned through \(N_z\) exchange spectra of a sample with NMR observable amounts of both cAMP-bound and \((R_p)\)-cAMPS-bound Rlaα-(119–244). In both cases, the \(N_z\) mixing period was 230 ms, and the relaxation delay between subsequent scans was 2 s. After acquisition of 128 scans per \(t_1\) transient, the \(N_z\) exchange spectra were processed using linear prediction. The \((\Delta\delta^{\text{eff}}_{\text{NOE}})^2 + (\Delta\delta^{\text{eff}}_{\text{15N}}/6.5)^2\) equation (39) was used to compute the compounded \(^1\text{H}, {^{15}}\text{N}\) chemical shift variations between the different states of Rlaα-(119–244) (i.e. X-bound versus free and X-bound versus cAMP-bound, where \(X = (S_p)\)- or \((R_p)\)-cAMPS).

**Relaxation Dispersion NMR**—A relaxation-compensated constant time (RC-CT) CPMG pulse sequence (40–42) was used to measure the \(^{15}\)N relaxation dispersions for backbone amides in the cAMP-, the \((R_p)\)-cAMPS-, and the \((S_p)\)-cAMPS-bound states of Rlaα-(119–244). The total CPMG length \(T_{\text{CPM}}\) was 93.3 ms with either 8 or 88 180°\(^{15}\)N pulses corresponding to CPMG field strengths \((\nu_{\text{CPMG}})\) of 43 and 472 Hz, respectively (40, 41). Fifteen interleaved replicate data sets were acquired and co-added for each CPMG field strength with 128 dummy scans and 16 scans per serial file separated by an interscan delay of 2.2 s. In addition, considering that at 700 MHz and at the CPMG radio frequency strength employed (3.1 kHz), \(^{15}\)N pulses are affected by significant offset effects, all CT-RC-CPMG experiments were acquired with three different \(^{15}\)N carrier frequencies (110, 119, and 127 ppm) to span the \(^{15}\)N spectral width using three narrow bands. The NMR relaxation dispersion \((\Delta\delta^{\text{eff}}_{\text{RC-CT}})\) was calculated as follows: \(\Delta\delta^{\text{eff}}_{\text{RC-CT}} = \delta_{\text{eff}}^{\text{43 Hz}} - \delta_{\text{eff}}^{\text{472 Hz}}\). Since \(K_{\text{eff}}(\nu_{\text{CPMG}}) = \frac{1}{T_{\text{CPM}}}\ln(\nu_{\text{CPMG}}/I_0)\)
(40, 41), where $I_{\text{CPMG}}$ and $I_{\alpha}$ are the cross-peak intensities with and without the CPMG periods, respectively, the equation for $\Delta R^2_{\text{eff}}$ can be recast as follows: $\Delta R^2_{\text{eff}} = (1/T_{\text{CP}}) \ln(I_{\text{CPMG}}/I_{\alpha})$ (29). For cross-peaks affected by overlap and/or relaxing too quickly to be detected in the CPMG spectra (i.e. Val$^{134}$, His$^{138}$, and Leu$^{399}$ in all three bound states), the $\Delta R^2_{\text{eff}}$ computation was not possible.

Other $^{15}$N NMR Relaxation Measurements—The $^{15}$N spin-lattice and spin-spin relaxation rates $R_1$ and $R_2$ as well as the $^{1}$H/$^{15}$N steady state NOEs were measured for the cAMP-, the $(R_\beta)$-cAMPS-, and the $(S_\beta)$-cAMPS-bound states of RIα-(119–244) using pulse sequences with water flip back pulses and sensitivity enhancement (34, 43–48). The $R_1$ relaxation rates were measured using relaxation delays of 100 ($\times$2), 200, 300, 400 ($\times$2), 500, 800, 900, and 1000 ms (where $\times2$ indicates duplicate spectra). The offset and duty cycle-compensated $^{15}$N $R_1$ CPMG experiments (49) were measured with a $v_{\text{CPMG}}$ of 472 Hz (40, 41) and CPMG relaxation delays of 8.48, 16.96, 25.4, 33.92, 42.4, 50.88, 59.36, 76.32, and 93.28 ms. The steady state $^{1}$H/$^{15}$N NOE values were measured from the ratio of peak intensities determined with and without proton saturation. For the NOE experiment, a 12-s recycle delay was used, including a 5-s proton saturation period (43, 45), whereas for the $R_1$ and $R_2$ pulse sequences, the recycle delay was 1.5 s. $^{15}$N heteronuclear single-quantum coherence two-dimensional NMR spectra were collected before and after each relaxation experiment to check the stability of the protein sample. The whole series of $R_1$ and $R_2$ relaxation rate measurements with different relaxation periods was collected in triplicate to average potential long term instabilities (39), and for the NOE experiments 10 sets of data were collected both with and without proton saturation. All replicate spectra were co-added before processing with NMRPipe (31). The relaxation rates and their errors were computed through relaxation fitting simulations with Sparky 3.111 (50), using 1000 iterations with a Gaussian and their errors were computed through relaxation fitting simulations with Sparky 3.111 (50), using 1000 iterations with a Gaussian

$R_1$ and $R_2$ as well as the $^{1}$H/$^{15}$N NOEs, whereas the $J(0)$ values are computed using the measured $^{15}$N relaxation rates and NOEs. For reference purposes, the Lorentzian spectral density of an isotropically diffusing rigid molecule was computed as follows: $J(\omega) = (2/5)\tau_\alpha/(1 + (\omega\tau_\alpha)^2)$.

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

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

RESULTS

Interaction Mapping of the Phosphorothioate $(S_\beta)$-cAMPS Agonist with CBD-A Using $N_\beta$ Spectroscopy

The binding of the cAMP agonist $(S_\beta)$-cAMPS (Fig. 1a) to RIα-(119–244) was mapped by $^1$H and $^{15}$N chemical shift changes measured through $N_\beta$ spectroscopy (Fig. 1c) (36–38). The resulting compounded chemical shift variations are reported in Fig. 2, a and c. Fig. 2a shows the $(S_\beta)$-cAMPS binding-induced compounded $^1$H and $^{15}$N chemical shift changes of RIα-(119–244), whereas Fig. 2c reports the compounded $^1$H and $^{15}$N ppm differences between the cAMP-bound and the $(S_\beta)$-cAMPS-bound forms of the same RIα construct. The pattern revealed by Fig. 2a is similar to what has been previously observed for cAMP binding to RIα-(119–244) (29). Statistically significant chemical shift changes are observed not only for the edges of the PBC spanning residues 199–211, where cAMP and its analogs dock (27), but also for two remote sites centered around Ile$^{163}$ and Asp$^{170}$. These two loci interact with Arg$^{209}$ in the PBC through hydrophobic and ionic interactions. As also seen for cAMP-binding (29), additional long range perturba-
FIGURE 1. Chemical structures of (Sp)-cAMPS (a) and (Rp)-cAMPS (b), respectively, and representative expansions from the longitudinal $^{15}$N$_z$ exchange spectra of Rα-(119–244) in the presence of cAMP and (Sp)-cAMPS (c) and of cAMP and (Rp)-cAMPS (d). The autopeaks of the cAMP-bound state are indicated by the residue number, and those of the analog-bound state are indicated by Sp- or Rp- appended to the residue number. The exchange cross-peaks arising from the transfer of magnetization between the cAMP- and the analog-bound states are connected by dashed lines. e and f, outline of the interaction networks radiating from the sulfur atoms of the phosphorothioate analogs of cAMP. e, (Sp)-cAMPS bound to site A of the regulatory subunit of PKA (Protein Data Bank code 1NE6) (27). f, (Rp)-cAMPS bound to site A of the regulatory subunit of PKA (Protein Data Bank code 1NE4) (27). In both panels the exocyclic sulfur and oxygen atoms of the phosphate are in yellow and red, respectively. The bound water molecule in the phosphate-binding cassette is highlighted in blue in e. Hydrogen bonds are depicted by black dashed lines. Selected residues are shown with red lines surrounded by their dotted van der Waals surfaces. The cAMP analogs are represented as CPK models. All images in e and f were created using the Molmol program (58).
Agonism and Antagonism in PKA

The similarity between cAMP and the (S_p)-cAMPS analog in terms of binding and allosteric effects is also confirmed by the comparison between the chemical shifts of cAMP-bound and of (S_p)-cAMPS-bound Rlx-(119–244) (Fig. 2c). No significant chemical shift change is observed for the majority of the residues in Rlx-(119–244), and the only significant variations are localized within the PBC and at residue Asn185 (Fig. 2c). Most of the PBC ppm differences reported in Fig. 2c are probably the result of the slight repositioning of (S_p)-cAMPS relative to cAMP within the Rlx-(119–244) binding pocket (27) caused by the steric effects of the (S_p)-cAMPS sulfur atom (van der Waals radius of 1.70 Å and P–S bond length of 1.95 Å) as compared with the cAMP oxygen atom (van der Waals radius of 1.35 Å and P–O bond length of 1.50 Å) (65). Furthermore, not only steric but also electronic effects should be taken into account for the amide of Ala210, which is hydrogen-bonded to the sulfur atom of (S_p)-cAMPS both directly and indirectly through a bridging water molecule (Fig. 1e) (27). The dual nature of the interaction between the Ala210 NH and the axial exocyclic heteroatom of the ligand explains the very dramatic chemical shift change detected for Ala210 upon replacement of oxygen with the bulkier and less electronegative sulfur in (S_p)-cAMPS (Figs. 1c and 2c). In addition, interresidue distances (Table S1) indicate that upon replacing cAMP with (S_p)-cAMPS, Ala210 is pushed away from the phosphorous atom and toward Gln186, which is in van der Waals contact with Ala210 and hydrogen bonds the amide of Asn185 (Fig. 2e). The backbone NH of Asn185 is therefore in an ideal position for sensing this relayed long range steric effect caused by (S_p)-cAMPS, explaining the significant chemical shift change observed for Asn185 in Fig. 2c.

Interaction Mapping of the Phosphorothioate (R_p)-cAMPS Antagonist with CBD-A Using Nε Spectroscopy

Similarly to (S_p)-cAMPS, the binding of the cAMP antagonist (R_p)-cAMPS (Fig. 1b) to Rlx-(119–244) was investigated using the 1H and 15N chemical shift changes obtained by Nε spectroscopy (Fig. 1d). The resulting compounded chemical shift variations are reported in Fig. 2b and d. The (R_p)-cAMPS binding-induced 1H and 15N chemical shift changes of Rlx-(119–244) are shown in Fig. 2b, whereas Fig. 2d shows the ppm differences between the cAMP-bound and the (R_p)-cAMPS-bound states of this cAMP-binding domain. Fig. 2b reveals a very distinct difference between the allosteric behavior of the (S_p)-cAMPS...
and \((R_p)\)-cAMPS. Although the binding of both phosphoro-thioate analogs causes perturbations at the PBC and at the more remote Ile\(^{163}\) and \(\alpha-B/\alpha-C\) interface regions (Fig. 2, \(a\) and \(b\)), only the \((S_p)\)-cAMPS agonist significantly perturbs the locus centered around Asp\(^{170}\) (Fig. 2, \(a\) and \(b\)). When the \((R_p)\)-cAMPS antagonist binds Rla-(119–244), the Asp\(^{170}\) region remains largely unaffected as compared with the free state.

Another clear difference between the \((S_p)\) and \((R_p)\)-cAMPS analogs is revealed by the inspection of Fig. 2d comparing the cAMP- and \((R_p)\)-cAMPS-bound states of Rla-(119–244). Fig. 2d shows that the replacement of the equatorial oxygen of bound cAMP with a sulfur atom causes significant perturbations not only at the PBC and at Asp\(^{170}\) but also in the allosteric sites centered at Ile\(^{163}\) and at the \(\alpha-B/\alpha-C\) boundary (29). These extensive long range perturbations caused by the \((R_p)\)-cAMPS/cAMP substitution (Fig. 2d) are in marked contrast with the trend observed for \((S_p)\)-cAMPS (Fig. 2c) and are accounted for by the more extensive Rla interaction network that radiates from the equatorial sulfur of \((R_p)\)-cAMPS (Fig. 1f) as compared with the axial sulfur of \((S_p)\)-cAMPS (Fig. 1e). The equatorial exocyclic heteroatom (i.e. oxygen or sulfur) in cAMP and its phosphorothioate analogs is anchored by multiple hydrogen bonds involving the amide of Ala\(^{202}\) and the guanidinium of Arg\(^{209}\). Perturbations at the equatorial position of cAMP-like ligands are therefore expected to cause extensive effects that propagate beyond the immediate PBC. For instance, Arg\(^{209}\) is ion-paired to Asp\(^{170}\) located at the N terminus of \(\beta_3\), and its methylene are also in van der Waals contact with the side chain of Ile\(^{163}\), which is positioned at the C terminus of \(\beta_2\) and is in turn adjacent to Val\(^{213}\) found within \(\beta_3\) (Fig. 1f). This interaction network explains the peak variations observed in Fig. 2d not only for the PBC but also for the Ile\(^{163}\) and Asp\(^{170}\) regions. Also, the significant ppm changes reported in the same panel (Fig. 2d) for the region at the \(\alpha-B/\alpha-C\) interface are consistent with the coupling between this site and the PBC occurring though the Glu\(^{200}\)/Arg\(^{241}\) salt bridge (66) and/or a hydrophobic hinge involving Ile\(^{204}\), Tyr\(^{229}\), and Met\(^{234}\) (12, 59).

Although the analysis of the compounded chemical shift variations (Fig. 2) clearly unveils significant differences between the \((S_p)\)-cAMPS agonist and the \((R_p)\)-cAMPS antagonist, a full understanding of their functional diversity warrants the investigation of their dynamic behavior as well. For reference purposes, we will first characterize the flexibility of the cAMP-bound form of Rla-(119–244). We will then analyze the changes in dynamics occurring when the axial or the equatorial exocyclic oxygens of cAMP are each replaced by a sulfur atom (i.e. when \((S_p)\)-cAMPS or \((R_p)\)-cAMPS, respectively, take the place of cAMP).

**Dynamic Features of cAMP-bound Rla-(119–244)**

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

Our relaxation data also indicate that, despite the relative rigidity of the \(\alpha-X_n\) and A-helices in the ps-ns time scale, the turn connecting them is affected by slow (ms-\(\mu\)) motions (Figs. 3, \(d\) and \(e\), \(4a\), and \(5\) and \(e\)). For instance, several residues between the \(\alpha-X_n\) and the A-helices are characterized by higher than average values observed for the \(R_1\) and \(R_2\) products (Fig. 3d) and for the reduced spectral densities at zero frequency (Fig. 4a). These \(R_1\) and \(R_2\) and \(J(0)\) variations are significantly larger than the fluctuations expected based on the anisotropy of the overall tumbling, as indicated by hydrodynamic modeling (Figs. 3d, \(4a\), \(S1\), and \(S2\)) and are therefore an indication of ms-\(\mu\) motions. The ms-\(\mu\) dynamics for the residues between the \(\alpha-X_n\) and the A-helices is also consistent with the secondary chemical shift results (Fig. 6) and with the line broadening beyond detection of the Val\(^{134}\), His\(^{138}\), and Leu\(^{139}\) cross-peaks. The flexibility in the region of the \(\alpha-X_n/\) A-helix loop is further supported by the loose packing of the \(\alpha-X_n/\)-helix suggested by the rapid HD exchange observed for the indole NH of Trp\(^{225}\) (data not shown), which in the x-ray structure of Rla-(91–376) (Protein Data Bank code 1RGS) is buried at the interface between the \(\beta\)-subdomain and the \(\alpha-X_n\)-helix (18).

The \(\beta\)-Subdomain—Our \(15N\) relaxation data (Figs. 3 and 4) point to a \(\beta\)-subdomain significantly less flexible than the \(\alpha\)-subdomain, in full agreement with previous hydrogen
JOURNAL OF BIOLOGICAL CHEMISTRY

Agonism and Antagonism in PKA

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

Despite the overall rigidity of the $\beta$-barrel, at least two main dynamic sites can be identified within the $\beta$-subdomain. The first $\beta$-subdomain dynamic site is Asp$^{170}$, which is located at the N terminus of $\beta_3$ and is affected by fast (ps-ns) local motions (Figs. 3c, 4c, and 5a and b). The second $\beta$-subdomain dynamic site includes the $\beta_{5,6}$ strands and the $\alpha$-B' helix within the adjacent PBC, defining a region characterized by slower (ms) and less local fluctuations (Figs. 3e, 4a, and 5c and d). The ps-ns motions of Asp$^{170}$ are identified by its decreased NOE (Fig. 3c) and its elevated $J(\omega_{H_1} + \omega_{N})$ value (Fig. 4c). The ms motions in the $\beta$-strands 5 and 6 and $\alpha$-B' helix regions are supported by the significant relaxation dispersion observed for residues Thr$^{190}$, Phe$^{190}$, Gly$^{199}$, and Tyr$^{205}$–Tyr$^{207}$ (Figs. 3e and 5c, and d). In addition, higher than average values are measured for both the $R_1R_2$ product (Fig. 3d) and the $J(0)$ (Fig. 4a) of Leu$^{203}$ in the $\alpha$-B' helix. Since these $R_1R_2$ and $J(0)$
Effects on the CBD-A Dynamics of the Oxygen to Sulfur Replacements at the Exocyclic Phosphate Positions of cAMP

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

Table 1 clearly shows that in both the β$_{2,3}$ loop and the PBC regions the average $J(0)$ values for (R)$_p$-cAMPS are significantly higher than those for cAMP and (S)$_p$-cAMPS. Considering that the overall tumbling correlation times and diffusional anisotropies of Rla-(119–244) do not differ significantly among the cAMP and phosphorothioate analog ligands (Table S2), the $J(0)$ trend revealed by Table 1 suggests the presence of enhanced ms-μs dynamics at the β$_{2,3}$ turn and the PBC sites in the (R)$_p$-cAMPS-bound state relative to the (S)$_p$-cAMPS- and cAMP-bound forms. These results are confirmed by the trend observed in Table 1 for the independently measured $\Delta R_{2}^{\text{eff}}$ (i.e. the average $\Delta R_{2}^{\text{eff}}$ values for (R)$_p$-cAMPS are significantly higher than those for cAMP and (S)$_p$-cAMPS both in the β$_{2,3}$ loop and in the PBC regions). The enhancement of the slow (ms-μs) dynamics specific to the (R)$_p$-cAMPS analog is highlighted in Fig. 7 by the orange ribbon shown for the β$_{2,3}$ turn and the PBC in (R)$_p$-cAMPS-bound Rla-(119–244).

No significant changes were identified in the fast (ps-ns) dynamic time scale as revealed by the absence of statistically meaningful variations in the average $J(\omega_H)$ variations among the three bound states for both the β$_{2,3}$ loop and the PBC sites (Table 1). Similar considerations apply to the analysis of the average $J(\omega_N)$ variations in the PBC (Table 1). However, a significant decrease in the mean β$_{2,3}$ loop spectral density at $\omega_N$ is detected for (R)$_p$-cAMPS relative to the other two bound states (Table 1 and Fig. S3a), supporting the conclusion that the $R_2$ analog promotes a shift in the dynamics of the β$_{2,3}$ turn toward slower (>ns) time scale motions consistent with the reported increase in the average $J(0)$ and $\Delta R_{2}^{\text{eff}}$ values.

DISCUSSION

Current Model of cAMP-dependent Allostery in PKA—The understanding of the molecular basis underlying the different functions of the (S)$_p$-cAMPS agonist and the (R)$_p$-cAMPS antagonist requires that our results be interpreted in the context of the current model for the cAMP-dependent allosteric control of PKA (29, 66), which is here briefly reviewed. The function of PKA is controlled by cAMP through an allosteric mechanism in which the cAMP signal propagates beyond the immediate PBC boundaries through multiple intramolecular signal pathways that cross the α/β-subdomain interface in CBD-A and ultimately perturb the R binding site for the catalytic subunit (C). Such pathways include both direct and indirect couplings between the PBC and the α-helical subdomain. Direct couplings involve a hydrophobic hinge cluster (12, 59) as well as a salt bridge between Glu$^{200}$ and Arg$^{241}$ (66), both con-

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Agonism and Antagonism in PKA

An Effective NMR Signature for cAMP Agonists and Antagonists—Our NMR results reveal that overall the effect of \((S_p^-)-\text{cAMPS}\) on R(CBD-A) resembles that of cAMP, whereas \((R_p^-)-\text{cAMPS}\) displays a different behavior from cAMP, both in terms of compounded chemical shift variations and dynamic profile. Specifically, a very distinct difference between \((S_p^-)\) and \((R_p^-)\)-cAMPS appears by considering their effects on the three allosteric hot spots previously identified in the model of the cAMP-dependent allostery (i.e. Ile\(^{163}\), Asp\(^{170}\), and the \(\alpha\)-B/C boundary)\(^{(29)}\). The comparative analysis of the compounded ppm changes reveals that \((S_p^-)-\text{cAMPS}\) perturbs these three allosteric sites according to a pattern very similar to that observed for cAMP. Unlike \((S_p^-)\)-cAMPS, the effect of \((R_p^-)\)-cAMPS on the same three sites is markedly different from that of cAMP; the Asp\(^{170}\) region remains essentially unaffected by the binding of \((R_p^-)-\text{cAMPS}\) to free R\(^{119-244}\), and the other two loci display chemical shifts that are different from those of both the cAMP-bound and the free forms of CBD-A. In other words, the diverse ppm trends observed for \((R_p^-)\) and \((S_p^-)\)-cAMPS (Fig. 2) at the allosteric triad sites (i.e. Ile\(^{163}\), Asp\(^{170}\), and the \(\alpha\)-B/C interface) define a clear NMR signature for the rapid identification of phosphorothioate cAMP antagonists and agonists (Fig. 2, red dotted rectangles). Although this result will facilitate the discovery of future phosphorothioate cAMP antagonists through NMR screening of cAMP analog libraries, further improvements in the targeted design of cAMP analogs require also that the functional differences between the \((R_p^-)\) and \((S_p^-)\)-analogues
be rationalized in terms of a molecular model. Such a model should account not only for the chain of perturbations revealed by the ppm variations but also for the dynamic and thermodynamic (28) differences observed between the (R<sub>p</sub>)- and (S<sub>p</sub>)-cAMPS-bound states of CBD-A.

A Molecular Model for the (R<sub>p</sub>)-cAMPS Antagonism—The chemical structures of cAMP and (R<sub>p</sub>)-cAMPS differ uniquely for the replacement of the exocyclic equatorial oxygen of cAMP with a sulfur atom (Fig. 1b). Although this might appear as a possibly minor isosbestic substitution, the introduction of sulfur not only induces local chirality at the phosphorous stereocenter but also results in steric and electronic effects. The former are primarily caused by the larger van der Waals radius of sulfur relative to oxygen and the accordingly longer P–S bond, whereas the latter are due to the reduced resonance and the increased localization on the sulfur of the phosphorothioate charge (18, 65). Furthermore, the multiple interactions that involve the equatorial exocyclic position (with Gly<sup>199</sup>, Glu<sup>200</sup>, and Arg<sup>209</sup> in Figs. 1f and 7b) relax and amplify the steric and electronic perturbations linked to the oxygen/sulfur replacement. Specifically, the phosphorothioate negative charge primarily localized on the equatorial sulfur atom of (R<sub>p</sub>)-cAMPS forms a very stable salt bridge with the guanidinium of Arg<sup>209</sup> (27) and competes with the carboxylate of Asp<sup>170</sup> for the neutralization of the guanidinium positive charge. As a result, the anchoring of the Asp<sup>170</sup> side chain to Arg<sup>209</sup> is weakened in the (R<sub>p</sub>)-cAMPS-bound form as compared with the cAMP-bound state (18), as also confirmed by the compound ppm changes, suggesting that the Asp<sup>170</sup> region is affected only by cAMP and (S<sub>p</sub>)-cAMPS binding but not by (R<sub>p</sub>)-cAMPS (Fig. 2). In other words, the Asp<sup>170</sup> site behaves similarly in the (R<sub>p</sub>)-cAMPS-bound form and in the free state of CBD-A, which is able to generate a stable R-C complex. The weakened Arg<sup>209</sup> anchoring of Asp<sup>170</sup> in the (R<sub>p</sub>)-cAMPS-bound form is also mimicked by the D170A mutation of the R-subunit (65). This mutant is nonallosteric, because the cross-talk between the cAMP- and the C-binding sites of CBD-A is compromised by the D170A mutation, but the affinities for both binding partners are preserved, leading to the formation of a stable cAMP-R(D170A)-C ternary complex (65). These similarities between the (R<sub>p</sub>)-cAMPS-R, free R, and cAMP-R(D170A) point to the existence of a stable (R<sub>p</sub>)-cAMPS-R-C ternary complex in which C is still inhibited even after (R<sub>p</sub>)-cAMPS binding (Fig. 7b), explaining why (R<sub>p</sub>)-cAMPS functions as a cAMP antagonist. In summary, the polarizing electronic effects of (R<sub>p</sub>)-cAMPS on the guanidinium of Arg<sup>209</sup> cause the obligatory intramolecular cAMP signaling pathway relayed by Asp<sup>170</sup> to be switched off, thus breaking the negative cooperativity between the cAMP- and C-binding sites and stabilizing the ternary inhibitory intermediate.

The similarity between the (R<sub>p</sub>)-cAMPS-R and free states of R, and consequently the stability of the ternary (R<sub>p</sub>)-cAMPS-R-C kinase-inhibitory complex, is further supported by the similar Gibbs free energies of unfolding measured for these two forms of R through urea denaturation (28). The ΔG<sub>u</sub> of cAMP-bound R-subunit decreases by more than 2 kcal/mol upon removal of cAMP, and the addition of (R<sub>p</sub>)-cAMPS to the

**TABLE 1**

| β<sub>p</sub>−β<sub>p</sub> loop (residues 163–171)<sup>a</sup> | 
| --- | --- | --- | --- |
| (R<sub>p</sub>)-cAMPS | cAMP | (S<sub>p</sub>)-cAMPS |
| J(0) × 10<sup>9</sup> (s/rad) | 3.22 ± 0.11 | 2.76 ± 0.05 | 2.77 ± 0.06 |
| J(100) × 10<sup>11</sup> (s/rad) | 2.27 ± 0.09 | 2.61 ± 0.05 | 2.56 ± 0.05 |
| J(100) + J(0) × 10<sup>11</sup> (s/rad) | 0.45 ± 0.15 | 0.42 ± 0.08 | 0.43 ± 0.08 |
| ∆G<sup>u</sup>(<sub>Δ</sub>) (s<sup>−1</sup>) | 1.87 ± 0.46 | 0.13 ± 0.15 | 0.10 ± 0.22 |

| PBC (residues 199–211)<sup>a</sup> | 
| --- | --- | --- | --- |
| (R<sub>p</sub>)-cAMPS | cAMP | (S<sub>p</sub>)-cAMPS |
| J(0) × 10<sup>9</sup> (s/rad) | 3.71 ± 0.14 | 3.43 ± 0.08 | 3.21 ± 0.08 |
| J(100) × 10<sup>11</sup> (s/rad) | 2.43 ± 0.15 | 2.63 ± 0.10 | 2.53 ± 0.10 |
| J(100) + J(0) × 10<sup>11</sup> (s/rad) | 0.38 ± 0.21 | 0.38 ± 0.14 | 0.40 ± 0.15 |
| ∆G<sup>u</sup>(<sub>Δ</sub>) (s<sup>−1</sup>) | 1.78 ± 0.68 | 1.22 ± 0.27 | 0.44 ± 0.50 |

<sup>a</sup> Only residues for which data is available for all three ligands were considered in the computations of the averages, in order to avoid introducing biases in the comparisons between different ligands.
free form does not cause any significant $\Delta G_{\text{unfolding}}$ change (28). This observation is fully consistent with the weakening of the Arg$^{209}$/Asp$^{170}$ salt bridge upon (R<sub>p</sub>)-cAMPs binding and with the effect of (R<sub>p</sub>)-cAMPs on the Ile<sub>163</sub> site as revealed by our ppm data (Fig. 2d). Unlike Asp$^{170}$, Ile<sub>163</sub> is predominantly affected by steric effects mediated by the adjacent Arg$^{209}$ methylenes. The (R<sub>p</sub>)-cAMPs-induced change in Ile<sub>163</sub> represents a significant perturbation for CBD-A, because Ile<sub>163</sub> belongs to a core group of buried hydrophobic amino acids that are well shielded from the solvent and are probably a key determinant of the global unfolding of CBD-A. The binding of (R<sub>p</sub>)-cAMPs causes therefore a partial global unfolding of CBD-A, which enhances its conformational plasticity similarly to the free state of CBD-A (28, 69, 70). The ensuing enhanced flexibility of the (R<sub>p</sub>)-cAMPs-bound state of R relative to its cAMP-bound form assists the $\alpha$-subdomain structural rearrangement required to recognize the C-subunit (20) and promotes the transition from the binary (R<sub>p</sub>)-cAMPs-R to the ternary (R<sub>p</sub>)-cAMPs-R-C complex.

The formation of the ternary (R<sub>p</sub>)-cAMPs-R-C complex is promoted not only by the global unfolding modes triggered through the Ile<sub>163</sub> pathway but also by the less global (ms-$\mu$s) motions that are specifically enhanced by (R<sub>p</sub>)-cAMPs and not by (S<sub>p</sub>)-cAMPs. These (R<sub>p</sub>)-cAMPs-specific motions are confined to the PBC and to the $\beta_2$-$\beta_3$ loop (Fig. 7b). The (R<sub>p</sub>) analog-enhanced ms-$\mu$s dynamics in the PBC is consistent with the extensive network of PBC interactions stemming from the equatorial exocyclic position of CAMP, which involves not only Arg<sup>209</sup> but also other critical residues, such as Ala<sup>202</sup> at the N terminus of the PBC $\alpha$-B helix (Fig. 1f). The dynamics at the $\beta_2$-$\beta_3$ loop is explained by the (R<sub>p</sub>)-cAMPs-induced perturbations at Ile<sub>163</sub> and Asp<sup>170</sup> located at the N and C termini of this region, respectively. Furthermore, the backbone CO and NH of Arg<sup>209</sup> hydrogen-bond the NH of Gly<sup>166</sup> and the CO of Asp<sup>167</sup> (18), respectively, further propagating the equatorial sulfuryl-induced effects from the PBC to the $\beta_2$-$\beta_3$ loop. Both the PBC and the $\beta_2$-$\beta_3$ regions include sites critical for C-binding (20) either directly through R-C contacts (i.e. the $\alpha$-B' helix) or indirectly through binding-coupled conformational changes (i.e. Asp<sup>170</sup>). It is therefore possible that the enhanced ms-$\mu$s dynamics observed for the PBC and the $\beta_2$-$\beta_3$ loci of the (R<sub>p</sub>)-cAMPs-bound state of R assists the initial recognition of the C-subunit by the (R<sub>p</sub>)-cAMPs-R binary complex, facilitating the formation of the ternary system that inhibits the kinase function.

It should be noted that the model proposed here focuses on CBD-A, whereas the full-length R-subunit is composed of two CBDs (CBD-A and CBD-B) (71), which communicate with each other allosterically. It is therefore possible that (R<sub>p</sub>)-cAMPs not only affects the internal allostery of CBD-A as discussed here, but it could also interfere with the allosteric cross-talk between CBD-A and CBD-B, as previously suggested (27). The effect of (R<sub>p</sub>)-cAMPs on CBD-A remains, however, critical to understanding how this analog is able to competitively inhibit PKA, as already pointed out (26, 72).

A Molecular Model for the (S<sub>p</sub>)cAMPs Agonism—As a result of the different local chirality between (R<sub>p</sub>)- and (S<sub>p</sub>)-cAMPs, the interaction networks propagating from the sulfur atoms of the (R<sub>p</sub>)- and (S<sub>p</sub>)-analogs are markedly different (Fig. 1, e and f, and Fig. 7, a and b). Unlike (R<sub>p</sub>)-cAMPs, the axial sulfur atom of (S<sub>p</sub>)-cAMPs does not interact with Arg<sup>209</sup>, and therefore it does not affect the two key allosteric sites linked to it (i.e. Asp<sup>170</sup> and Ile<sub>163</sub>) differently from cAMP. The (S<sub>p</sub>)-cAMPs steric and electronic effects are mainly localized on Ala<sup>210</sup> (2c). The only long range perturbation caused by the cAMP/(S<sub>p</sub>)-cAMPs replacement is observed for Asn<sup>185</sup> (Fig. 2c), which is caused by a steric effect on Ala<sup>210</sup> relayed by Gln<sup>165</sup> to the proximal Asn<sup>185</sup>. This residue is located in the loop between strands $\beta_4$ and $\beta_5$, a region characterized by the highest sequence variability among different cAMP-binding domains (73) and not involved in the binding of cAMP or C. The perturbations caused by the sulfur-to-oxygen replacement at the axial exocyclic position are therefore not functionally relevant and explain the similar allosteric profile observed for cAMP and (S<sub>p</sub>)-cAMPs, rationalizing the (S<sub>p</sub>)-cAMPs agonism. Consistent with the agonist function of (S<sub>p</sub>)-cAMPs, it is anticipated that the ternary (S<sub>p</sub>)-cAMPs-R-C intermediate (Fig. 7a) is significantly less stable than the (R<sub>p</sub>)-cAMPs-R-C-inhibitory complex (Fig. 7b). It should, however, be noted that the ternary intermediate could still be formed, at least transiently and at low concentrations, even in the case of cAMP and of the functionally homologous (S<sub>p</sub>)-cAMPs (Fig. 7a). This hypothesis is supported by the dynamics of the cAMP-bound state of CBD-A, as explained below, as well as by the recent finding that cAMP still binds the PKA holoenzyme with a micromolar affinity (74, 75).

Functional Relevance of the Residual Dynamics in the CAMP-bound State—The functional relevance of the CBD-A dynamics in its CAMP-bound state is evaluated in relation to the R-C binding sites. The recently solved structure of the R(CBD-A)-C complex (20) has revealed three key R(CBD-A)/C interaction sites that map to the $\alpha$-B’ helix, the C-helix, and the $\alpha$-X<sub>n</sub>/A-helix turn (Fig. 5, black dashed contours). All three R-C interaction regions within CBD-A match surprisingly well with the dynamic “hot spots” observed for the CAMP-bound state of R-lol-(119–244) (Fig. 5, a–d). The $\alpha$-X<sub>n</sub>/A-helix loop and the PBC are two of the most significant clusters subject to ms-$\mu$s motions (Figs. 3, b, d, and e, 4a, and 5, c and d), whereas the C-helix is intrinsically dynamic and highly conformationally heterogeneous in the ps-ns time scale (Figs. 3, a–c, 4, a–c, 5, a and b, and 6), in full agreement with recent molecular dynamics simulations (66, 76). The structure of the R(CBD-A)-C complex (20) has also revealed that significant local conformational differences exist between the C- and CAMP-bound states of CBD-A for loci not involved in direct interactions with C. These sites of local conformational divergence between the C- and CAMP-bound states of CBD-A are localized in the vicinity of Asp<sup>170</sup> and at the C terminus of the $\alpha$-X<sub>n</sub>/A-helix (Fig. 5, red dashed contours) and correlate well with those that are dynamic in the ms-$\mu$s time scale (Fig. 5, c and d) and, in the case of Asp<sup>170</sup>, in the ps-ns time frame as well (Fig. 5, a and b).

The residual dynamics of the CAMP-bound state of CBD-A may reflect at least in part its intrinsic flexibility, as is probably the case for the loop region between the $\beta$-stands 4 and 5, which is known to be poorly structurally defined in several CBDs of R-isofoms (73). However, the overall correlation observed between the dynamic hot spots of R-lol-(119–244) and the sites of C binding and C binding-induced conformation changes
(Fig. 5) suggests (77) that the residual dynamics observed for the cAMP-bound state of CBD-A may assist the early recognition of the C-subunit even before cAMP is released from the R-subunit, leading to the transient formation of the cAMP-R-C ternary intermediate, as hypothesized above. Consistent with this hypothesis, the residual dynamic hot spots identified for the cAMP-bound CBD-A may facilitate the reversible toggling of the R-subunit between the cAMP-bound and the C-bound states, namely the reversible transition from the active to the inactive state of protein kinase A. Considering that the preservation of dynamics even after ligand binding has been recently reported also for other systems (67, 78), it is possible that the residual plasticity of the ligand-bound state represents a general signaling mechanism that has evolved to ensure the reversibility in the binding to mutually exclusive partners.

CONCLUSIONS

We have mapped by N2 exchange NMR spectroscopy and 15N relaxation measurements the interactions and the dynamics of the binary R(CBD-A)-(S)p-cAMPS and R(CBD-A)-(R)p-cAMPS complexes. The comparative analysis of these phosphothioate-bound forms has revealed significant allosteric and dynamic differences between the (R)p-cAMPS- and cAMP-bound states, whereas the (S)p-cAMPS-bound form behaves similarly to the cAMP-R(CBD-A) complexes, in agreement with the functional diversity between the (R)p- and (S)p-cAMPS agonism and agonism based on steric and electronic effects on two key allosteric relay sites, Ile163 and Asp170. The perturbations caused by (R)p-cAMPS at these sites not only result in increased ms-μs flexibility for the local motions confined to the PBC and to the proximal β2-β3 loop; they also enhance global partial unfolding modes. Both effects promote the conformational plasticity at the α/β-subdomain interface and therefore the stabilization of a ternary inhibitory (R)p-cAMPS-R-C complex. In the presence of (S)p-cAMPS or cAMP, which do not affect the Ile163 and Asp170 allosteric sites as (R)p-cAMPS, this ternary intermediate is less stable, but it can still form at least transiently and with lower populations, as suggested by the correlation between several sites of residual ms-μs and ps-ns dynamics in the cAMP- and (S)p-cAMPS-bound states of R(CBD-A) and the loci of C-binding and of C-induced local conformational change. Such correlation points to an ancillary role of the residual cAMP-bound state dynamics to assist the early recognition of free C by R even before cAMP dissociates from R, supporting the transient existence of the ternary complex involving cAMP, R and C and facilitating the transition from the “on” to the “off” state of PKA. These considerations suggest a general signaling mechanism adopted and conserved by proteins with mutually exclusive binding sites to ensure reversibility in the interactions with their molecular partners.

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

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