Cyclic AMP- and (R<sub>p</sub>)-cAMPS-induced Conformational Changes in a Complex of the Catalytic and Regulatory (RIα) Subunits of Cyclic AMP-dependent Protein Kinase*[][]

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We took a discovery approach to explore the actions of cAMP and two of its analogs, one a cAMP mimic ([S<sub>p</sub>]-adenosine cyclic 3':5'-monophosphorothioate ([S<sub>p</sub>]-cAMPS)) and the other a diastereoisomeric antagonist ([R<sub>p</sub>]-cAMPS), on a model system of the type α cyclic AMP-dependent protein kinase holoenzyme, RIα(91–244)C-subunit, by using fluorescence spectroscopy and amide H/H exchange mass spectrometry. Specifically, for the fluorescence experiments, fluorescein maleimide was conjugated to three cysteine single residue substitution mutants, R92C, T104C, and R239C, of RIα(91–244), and the effects of cAMP, ([S<sub>p</sub>]-cAMPS, and ([R<sub>p</sub>]-cAMPS on the kinetics of R-C binding and the time-resolved anisotropy of the reporter group at each conjugation site were measured. For the amide exchange experiments, ESI-TOF mass spectrometry with pepsin proteolytic fragmentation was used to assess the effects of ([R<sub>p</sub>]-cAMPS on amide exchange of the RIα(91–244)C-subunit complex. We found that cAMP and its mimic perturbed at least parts of the C-subunit interaction Sites 2 and 3 but probably not Site 1 via reduced interactions of the linker region and αC of RIα(91–244). Surprisingly, ([R<sub>p</sub>]-cAMPS not only increased the affinity of RIα(91–244) toward the C-subunit by 5-fold but also produced long range effects that propagated through both the C- and R-subunits to produce limited unfolding and/or enhanced conformational flexibility. This combination of effects is consistent with ([R<sub>p</sub>]-cAMPS acting by enhancing the internal entropy of the R-C complex. Finally, ([R<sub>p</sub>]-cAMPS-induced increase in affinity of RIα(91–244) toward the C-subunit indicates that ([R<sub>p</sub>]-cAMPS is better described as an inverse agonist because it decreases the fractional dissociation of the cyclic AMP-dependent protein kinase holoenzyme and in turn its basal activity. *Molecular & Cellular Proteomics 9:2225–2237, 2010.

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Cyclic AMP-dependent protein kinase (PKA) plays a crucial role in a plethora of cellular functions. All isoforms of PKA are composed of two catalytic (C) subunits and homodimeric regulatory (R) subunits (1–3). As the name implies, cAMP is a major PKA regulator (4). Much progress has been made in the last decade in delineating the molecular basis of action of cAMP. An important tactic in this endeavor has been through the comparison of the effects of cAMP with those of two phosphorothioate cAMP analogs: ([S<sub>p</sub>]-cAMPS (a CAMP mimic) and ([R<sub>p</sub>]-cAMPS (an antagonist and a diastereoisomer of ([S<sub>p</sub>]-cAMPS). Although the importance of geometry of the sulfur substitution is critical in determining the pharmacological properties of the two phosphorothioate cAMP analogs, the molecular basis for this behavior is not fully understood. To date, these comparisons have only been made using either wild-type or truncated mutants of the type α regulatory subunit (RIα) that are free in solution, not complexed to the C-subunit. X-ray spectroscopic examination of ligand-bound RIα(92–379) complexes reveals few differences between ligand-bound complexes, but the ([R<sub>p</sub>]-cAMPS complex is structurally “looser” with higher thermal factors than complexes formed with either cAMP or ([S<sub>p</sub>]-cAMPS (5). This is consistent with the observation that both cAMP and ([S<sub>p</sub>]-cAMPS, but not ([R<sub>p</sub>]-cAMPS, raise the urea concentration required for wild-type RIα unfolding (6). Further insight into the structural basis of cAMP action stems from NMR spectroscopic comparison of the effects of ([R<sub>p</sub>]-cAMPS, cAMP, and ([S<sub>p</sub>]-cAMPS on chemical shifts and 15N relaxation of the RIα(119–244) mutant (7). In addition to producing fewer significant chemical shift changes than either cAMP or ([S<sub>p</sub>]-cAMPS, ([R<sub>p</sub>]-cAMPS binding is associated with enhanced millisecond to microsecond time scale backbone motions of a β-turn (β2,3 loop) and around the phosphate-binding cassette (PBC) (7).

The abbreviations used are: PKA, cyclic AMP-dependent protein kinase; C-subunit, catalytic subunit of cAMP-dependent protein kinase; R-subunit, regulatory subunit of cAMP-dependent protein kinase; RIα, type α regulatory subunit; RIα(91–244), deletion mutant of type α isoform of the R subunit; CNB, cyclic nucleotide binding; FM, fluorescein maleimide; PBC, phosphate-binding cassette; cAMPS, adenosine cyclic 3':5'-monophosphorothioate.
cAMP- and (Rp)-cAMPS-induced Conformational Changes

Further insight into the molecular basis of actions of cAMP and its analogs should come from the analysis of ligand-bound R-C complexes. Unfortunately, the large size of even the heterodimeric R-C complex (~95 kDa) and the difficulty of preparing (Rp)-cAMPS-R-C-subunit crystals currently preclude the use of both NMR spectroscopy and x-ray crystallography. Consequently, we took two alternative lower resolution approaches to this issue. One approach involves the use of site-directed labeling combined with fluorescence spectroscopy to examine both the effects of cAMP and its analogs on solvent exposure/conformational flexibility of R-C-subunit binding kinetics and on the conformational dynamics of RIα(91–244). RIα(91–244) includes the “A” cyclic nucleotide binding (CNB) domain, the pseudosubstrate, and linker domains and represents the minimal segments necessary for high affinity C-subunit binding (Fig. 1) (8). The other approach involves an examination of the effects of cAMP and its analogs on solvent exposure/conformational flexibility of RIα(91–244):C-subunit complex using H/2H amide exchange measured with a combination of mass spectrometry (ESI-Q-TOF) and proteolytic fragmentation. In the first approach, fluorescein maleimide (FM) was conjugated to three cysteine substitution mutants with the substitution sites located near or within the pseudosubstrate sequence, the linker domain, or αC (R92C, T104C, and R239C, respectively) of RIα(91–244) (Fig. 1). The time-resolved fluorescence anisotropy results suggest that cAMP and (S50)-cAMPS reduce the interaction of the RIα linker domain and αC with the two peripheral R-C interaction sites on the C-subunit (so-called Sites 2 and 3) without affecting the interaction of the pseudosubstrate sequence with the active site cleft (so-called Site 1). Because of limitations of the amide H/2H exchange experiments, only the effects of (Rp)-cAMPS on H/2H amide exchange in RIα(91–244):C-subunit complex could be investigated. The results showed that (Rp)-cAMPS induces a relatively widespread increase in amide exchange, indicating limited unfolding and/or enhanced conformational flexibility that is propagated almost globally through the C-subunit and, at least, part of RIα. These conformational changes were accompanied by a 5-fold increase in the affinity of RIα(91–244) toward C-subunit, suggesting that, at least, some of the (Rp)-cAMPS effects are mediated by an increase in internal entropy. Finally, the (Rp)-cAMPS-induced increase in R-C affinity indicates that (Rp)-cAMPS is better described as an inverse agonist because the basal activity of the PKA holoenzyme should be decreased by (Rp)-cAMPS.

EXPERIMENTAL PROCEDURES

Materials—ATP, cAMP, and MOPS were obtained from Sigma-Aldrich. (S50)-cAMPS and (Rp)-cAMPS triethylamine were obtained from Biolog (Bremen, Germany). Nickel-nitrilotriacetic acid resin was obtained from Qiagen (Chatsworth, CA). Immobilized pepsin cartridges (Poroszyme) were obtained from Applied Biosystems (Redwood City, CA). Immobilized cobalt affinity columns (Talon, Clontech) were used for purification of hexahistidine-tagged PKA C-subunit following the manufacturer’s specifications. Deuterium oxide (99.9% deuterium) was obtained from Cambridge Isotopes (Andover, MA). Trifluoroacetic acid (TFA) and acetonitrile were from Fisher Scientific. PD10 columns were from GE Healthcare. All other chemicals were at least reagent grade.
Preparation of Rlu(91–244) Mutants and C-subunit—Three cysteine substitution mutants (R92C, T104C, and R239C) of Rlu(91–244) subunit were prepared as described previously (9). Hexahistidine-tagged murine PKA Cα-subunit was expressed in *Escherichia coli* and purified as described previously (10). Isotzy I was used for all experiments. The holoenzyme of PKA was prepared as described earlier using 50 mM MOPS, pH 7.0, 1 mM DTT, 0.2 mM ATP, and 2 mM MgCl₂ (9).

**Fluorescein Maleimide Labeling**—The R-subunit samples (12.5–25 nmol) were initially buffer-exchanged by elution through a G-25 column (1.5 × 7 cm) equilibrated with buffer (50 mM MOPS, 50 mM NaCl, pH 7.0). The protein fractions were pooled, and the concentrations of the pooled samples were determined with the Bradford assay (11). Unless stated otherwise, the protein concentrations of the reaction mixtures ranged between 3 and 6 μM. The reactions were allowed to proceed for 1 h at room temperature, protected from light, and then eluted through a PD10 column (2.5 × 6 cm) followed by a Sephacryl S-200 column (2.5 × 7 cm), both equilibrated with buffer A (50 mM MOPS, 50 mM NaCl, 1 mM DTT, pH 7.0) at room temperature. Fluorescein emission (excitation at 470 nm and emission at 525 nm) from the column fractions was measured, and the fluorescent fractions with retention times that corresponded to unmodified R-subunit were pooled. Aliquots of the pooled fractions were subjected to gel electrophoresis under denaturing conditions (12% SDS-PAGE), and the fluorescent bands were visualized with a mineral lamp to assess the presence of unconjugated fluorescein in the samples.

**Phosphotransferase Assay**—The activity of the labeled R mutants was evaluated by assessing their ability to inhibit the phosphotransferase activity of recombinant C-subunit using the method of Cook et al. (12) with Kemptide as a substrate.

**Determination of Stoichiometry of Labeling**—The stoichiometry of FM-labeled R was determined spectrophotometrically by substitution of the measured absorbance values at 497 nm (A₄₉₇) and the protein concentration [R] determined with the Bradford assay (11) into the following expression.

\[
[\text{fluorescein}]/[R] = (A₄₉₇/83,000)/[R] \quad (\text{Eq. 1})
\]

**Formation of Rlu(91–244)-C Complexes**—The labeled and unlabeled Rlx mutants (1 μM) were combined with a 1.2-fold molar excess of recombinant C-subunit and then dialyzed (cutoff, 30 kDa) overnight at 4 °C against 3 × 1-liter changes in buffer B (50 mM MOPS, 50 mM NaCl, 1 mM DTT, 2 mM MgCl₂, 0.2 mM ATP, pH 7.0). Free C- and R-subunits were separated from the Rlu(91–244)-C complexes by elution through a Sephacryl S-200 column (1.5 × 25 cm) using buffer B.

**Stopped-flow Kinetic Measurements**—Stopped-flow experiments were performed with an Applied Photophysics SX.18MV (Leatherhead, UK) stopped-flow spectrophotofluorometer. The samples were excited at 460 nm, and an Omega interference filter (510DF23) was used to select the fluorescence signal. The second-order association rate constants for binding of C-subunit to the Rlu(91–244) conjugate was determined from the slope of plots of the observed rate of fluorescence change versus final C-subunit concentration. The first-order rate constant of R-C dissociation was determined by mixing the preformed FM-R92C Rlu(91–244) complexes with a 10-fold excess of unlabeled Rlu(91–244) and fitting the tracings to a single exponential equation. All samples were prepared in buffer containing MgATP (50 mM MOPS, 50 mM NaCl, 1 mM DTT, 2 mM MgCl₂, 0.2 mM ATP, pH 7.0). Equilibrium dissociation constants (Kᵥ values) were determined as a ratio of the dissociation to association rate constants.

**Time-resolved Fluorescence Anisotropy**—Emission anisotropy was determined as described previously (13). Unless stated otherwise, emission anisotropy decay was analyzed with the impulse reconvolution method implemented in the DASE™ software package from HORIBA Jobin Yvon IBH Ltd. (Glasgow, UK) described elsewhere (14). Briefly and simply, this approach splits the analysis into two steps: analysis of the total emission decay, S(t), from the vertically, Iᵥ(t), and orthogonally, Iᵦ(t), polarized emission components followed by analysis of the vertical/perpendicular difference emission decay, D(t). S(t), free of anisotropy effects, is given by the expression

\[
S(t) = Iᵥ(t) + G \cdot 2Iᵦ(t) \quad (\text{Eq. 2})
\]

and is analyzed as a biexponential function. G is a measure of the instrumental polarization bias (0.995). D(t), which includes both fluorescence and anisotropy parameters, is given by the following expression.

\[
D(t) = Iᵥ(t) - G \cdot Iᵦ(t) \quad (\text{Eq. 3})
\]

D(t) is deconvolved with the results from the S(t) analysis as a constraint, yielding the following expression.

\[
r(t) = βᵦ \cdot \exp(-t/τᵦ) + βᵥ \cdot \exp(-t/τᵥ) \quad (\text{Eq. 4})
\]

Here, βᵦ and βᵥ are the amplitudes of the anisotropy at time 0 for the fast and slow anisotropy decay processes, respectively. τᵦ and τᵥ are the fast and slow rotational correlation times of the anisotropy decay, respectively. τᵥ usually yields an estimate of the whole-body rotational correlation time when it is less than about 5 times the emission lifetime and when the rate of the segmental motions around the site of reporter group conjugation greatly differ from the rate of the whole-body diffusion. A nonassociative model was assumed where the emission relaxation times are common to all the rotational correlation times. Goodness of fit was evaluated from the values of the reduced χ² and by visual inspection of the weighted residual plots. All time-resolved anisotropy measurements were performed with samples suspended in buffer B at 22 °C.

**Preparation of Rlu(91–244)-C-subunit Complexes for H²H Exchange Analysis**—Rlu(91–244)-C-subunit complexes were concentrated to ~150 μM using concentrators (Amicon, Millipore) (molecular mass cutoff, 10 kDa), and where appropriate, (Rᵥ)-cAMPS was added to a concentration of 10 mM. This enabled the final concentration of (Rᵥ)-cAMPS to be 1 mM under deuterium exchange conditions.

**Amide Hydrogen-Deuteron Exchange ESI-Q-TOF Mass Spectrometry**—The C-Rlu(91–244) R92C protein complexes (109 μM) in the absence and presence of 1 mM (Rᵥ)-cAMPS were allowed to exchange with deuterated buffer solutions by 10-fold dilutions of protein samples in deuterated 50 mM MOPS, 50 mM NaCl, 2 mM MgCl₂, 0.2 mM ATP, 1 mM DTT, pH 7.0 (deuterated buffer B). The reactions were then quenched after timed intervals of 30 s, 1 min, 2 min, 5 min, and 10 min by placement on ice and the addition of 180 μL of 0.2% trifluoroacetic acid, pH 2.5. Undeuterated samples were included as a negative control. One sample was allowed to exchange with deuterated buffer B for 24 h to allow for complete deuteration of solvent-exposed regions of the protein. This was used to calculate back-exchange under our experimental conditions. 50 μL of the reaction samples were digested through an on-line Poroszyme immobilized pepsin cartridge (Applied Biosystems) before passing through an ACQUITY UPLC BEH C₁₈ column. The reaction samples were then analyzed on a Waters Synapt high definition mass spectrometry (HDMS) system (Waters). Both the pepsin cartridge and C₁₈ column were housed in a refrigerated module at 2 °C to minimize deuterium back-exchange during analysis (15, 16).

Peptide identification was achieved by Protein Lynx Global Server (PLGS) (v2.4) search software (Waters) using a database containing a primary sequence of PKA Rlu(91–244) and C-subunits. Cleavage peptides by nonspecific proteases with a mass tolerance of 10 ppm were used to sequence the peptides. Continuous instrument calibration was carried out with Glu-fibrinogen peptide at 100 fmol/μL. Deu-
terium exchange quantitation was by HX-Express software (17). A control experiment was carried out to calculate the deuterium back-exchange loss during the experiment by incubating ligand-free Rlu (91–244) with deuterated buffer A for 24 h at room temperature (20 °C). Even after extended deuteration, Rlu (91–244) still showed some solvent-inaccessible and ordered regions that were not completely deuterated. For an accurate measurement of back-exchange loss, we therefore focused only on peptides from within highly solvent-exchangeable regions of the protein, identified as those regions that show greater relative exchange at shorter time points (10-min exchange). The region in Rlu (91–244) spanning residues 111–130 is a highly solvent-exchangeable region (10), and all five overlapping peptides used for calculations of back-exchange span this region and showed nearly complete exchange in ligand-free Rlu (91–244) following 10-min deuteration exchange and would represent fully deuterated samples following 24-h exchange. An example of the effects of deuteration following 24-h exchange on a fragment spanning residues 111–126 (m/z = 632.70) is illustrated in supplemental Fig. 9. The average deuterium back-exchange of 32.8 ± 1.6% was calculated from average back-exchange values for five peptides: Rlu (111–123) (m/z = 547.65) (back-exchange, 34.4%), Rlu (111–119) (m/z = 559.34) (back-exchange, 33.7%), Rlu (111–126) (m/z = 632.70) (back-exchange, 30.4%), Rlu (111–119) (m/z = 595.34) (back-exchange, 33.7%), and Rlu (112–126) (m/z = 578.35) (back-exchange, 32.0%). All deuterium exchange values reported were corrected for a 32.7% back-exchange by multiplying the raw centroid values by a multiplication factor of 1.49.

RESULTS

Preparation, Labeling, and Characterization of Rlu (91–244) Mutants—The three cysteine substitution mutants were overexpressed and purified to greater than 95% as assessed by visual inspection of Coomassie-stained SDS-PAGE gel and FPLC profiles. Mass spectrometry of each mutant was consistent with the expected fragmentation masses by peptide mass fingerprinting by MALDI-TOF analysis of the desired mutant (data not shown), and each mutant was as equally effective as Rlu (91–244) at inhibiting the phosphotransferase activity of the wild-type C-subunit (data not shown) (9). Denaturing SDS-PAGE analysis of the fluorescein conjugates showed no detectable nonconjugated probe, and the stoichiometry of fluorescein conjugation was ~28, ~24, and ~40% for the FM-R92C, FM-T104C, and FM-R239C Rlu (91–244) conjugates, respectively.

Effects of cAMP, (Rp) and (Sβ) on Kinetics of C-subunit Binding to Rlu (91–244) —To gain a better understanding of the effects of (Rp)-cAMPS or R-C interactions and to determine the reactant concentrations required to maintain R- and C-subunits in a heterodimeric state in the presence of cAMP or (Sβ)-cAMPS, the association and dissociation rate constants for R-C binding were determined in the presence and absence of cAMP, (Rp)-cAMPS, and (Sβ)-cAMPS. Because FM-R92C conjugate binding to C-subunit is associated with a relatively large and unambiguous change in fluorescence (~40% decrease) compared with the other conjugated mutants (9), the FM-R92C conjugate was utilized to assess the effects of cAMP and two phosphorothioate analogs on the kinetics of C-subunit binding. Representative stopped-flow tracings associated with the rapid mixing of the FM-R92C conjugate with various concentrations of C-subunit in the absence and presence of cAMP (10 μM), (Rp)-cAMPS (100 μM), and (Sβ)-cAMPS (100 μM) are illustrated in Fig. 2. The fluorescence tracings were well fit to a single exponential equation, and the bimolecular association rate constants were determined from the slopes of the plots of the observed rates for fluorescence change versus final concentration of C-subunit. The values of the association rate constants were essentially the same, ranging between 1.7 and 2.3 × 10^7 M⁻¹ s⁻¹ (Table I), indicating that the binding of cAMP and its analogs does not affect the association process. The effects of cAMP and its analogs on the time course of dissociation of the FM-R92C-C-subunit complex on the other hand were analog-dependent. Fig. 3, A and B, illustrate representative stopped-flow tracings associated with the rapid mixing of preformed FM-R92C-C-subunit complexes (100 nM) with excess unlabelled Rlu (91–244) (1 μM) in the absence and presence of cAMP (10 μM), (Rp)-cAMPS (100 μM), or (Sβ)-cAMPS (100 μM). Fitting the experimental tracings to a single exponential equation yielded a unimolecular dissociation rate constant for the control sample of 3.8 × 10⁻² s⁻¹, essentially identical to what we observed previously (9). As expected, the presence of cAMP or (Sβ)-cAMPS was associated with an increase in the dissociation rate constant (150–200-fold; Table I). The presence of (Rp)-cAMPS was associated with an unexpected result. Because (Rp)-cAMPS is generally regarded as a competitive antagonist, we expected it to
mixed with excess unlabeled RI

Thr104, and Arg239 was studied. These residues were chosen (10
concentrations of C-subunit in the absence and presence of cAMP
dissociation rate constants for C-subunit binding to FM-R92C RI
Effect of cAMP, (Sp)-cAMPS, and (Rp)-cAMPS on association and
c-subunit—

TABLE I

|            | $k_+^a$ | $k_-^b$ | $K_a^c$ |
|------------|---------|---------|---------|
| Control    | 2.0 ± 0.12 × 10^7 | 3.0 ± 0.1 × 10^-3 | 1.9 × 10^-10 |
| cAMP       | 1.7 ± 0.11 × 10^-7 | 0.55 ± 0.05 | 3.2 × 10^-8 |
| (R91)-cAMPS| 1.9 ± 0.17 × 10^-7 | 7.5 ± 0.3 × 10^-4 | 4.0 × 10^-11 |
| (S91)-cAMPS| 1.9 ± 0.16 × 10^-7 | 0.75 ± 0.02 | 4.0 × 10^-8 |

$^a$ Bimolecular association rate constants determined from rapid mixing of FM-labeled Rlx(91–244) mutants (100 nM) with various concentrations of C-subunit in the absence and presence of cAMP (10 μM), (R91)-cAMPS (100 μM), or (S91)-cAMPS (100 μM) under pseudo first-order conditions ($n = 4$).

$^b$ Unimolecular dissociation rate constants determined by rapidly mixing excess unlabeled Rlx(91–244) with preformed Rlx(91–244)-C complex (100 nM) of FM-labeled Rlx(91–244) conjugates and C-subunit in the absence and presence of cAMP (10 μM), (R91)-cAMPS (100 μM), or (S91)-cAMPS (100 μM) ($n = 3$).

$^c$ Equilibrium dissociation constants determined by dividing the unimolecular dissociation by the bimolecular association rate constants.

have no significant effect on FM-R92C-C-subunit dissociation, but surprisingly, (R91)-cAMPS slowed the dissociation rate by 5-fold, increasing the affinity of FM-R92C for C-subunit by an equal amount (Table I).

Anisotropy Decay of FM Conjugates Free and Bound to C-subunit—To lay a foundation for analyzing the effects of cAMP and its analogs on solution structure and dynamics of C-subunit-bound Rlx(91–244), the time-resolved anisotropy of FM conjugated to substituted cysteines at residues Arg92, Thr104, and Arg239 was studied. These residues were chosen to be near but not part of the C-subunit binding surface (Fig. 1). Representative anisotropy decays of the unliganded con-

figured mutants free in solution and complexed to the C-subunit are illustrated in Fig. 4, and the fitting parameters are summarized in Table II. Although the interpretation of anisotropy decay is frequently problematic, in the present case, the results are largely consistent with crystallographic and NMR structural results. The typical anisotropy decay from a reporter group conjugated to a side chain of a globular protein fits reasonably well to a biexponential function with the time zero anisotropy ($\beta_1 + \beta_2$) at a value less than the fundamental anisotropy for the reporter group due to rapid, unresolved depolarization of torsional tether arm motion. (The fundamental anisotropy of FM equals 0.34.) The slowest rotational correlation time ($\phi_{slow}$) is usually attributable to global whole-body rotational diffusion. The amplitude of this slow depolarization process ($\beta_2$) is a complex function of the maximum angular excursion of diffusion in a cone associated with both tether arm and $\alpha$-carbonyl backbone motion adjacent to the site of conjugation and is inversely related to the maximum excursion angle of the reporter group (18, 19). The faster correlation time ($\phi_{fast}$) is a complex function of both diffusion rate and the maximum angular excursion (assuming diffusion in a cone) of primarily $\alpha$-carbonyl backbone diffusion around the site of conjugation. Unresolved anisotropy decay, i.e. the difference between the fundamental and time zero anisotropy, most likely reflects subnanosecond tether arm torsional motions.

For the FM-conjugated mutants free in solution, the slow rotational correlation times ranged between 7.6 and 13.0 ns, which is about what is predicted from the Stokes-Einstein equation for a 15-kDa sphere (5.9 ns) or that estimated by the Hydropro computer program (version 7.c) (20) (10.9 ns) using the atomic coordinates of the x-ray structure of Rlx(91–244). The amplitudes of the slow depolarization processes ($\beta_2$) were in the rank order R92C < T104C < R239C and ranged between 0.130 and 0.173. This rank order is consistent with what would be expected from visual inspection of the crystal
structure of Rlα(91–244) with the unstructured N-terminal R92C position being the most unhindered followed by the T104C at 17 residues N-terminal from the structured αN (starting at Lys121) followed by residue R239C near the C terminus of αC. The fast rotational correlation times are similarly ranked order with the R92C having the fastest correlation times (0.9 ns) followed by the T104C (2.1 ns) and R239C (2.4 ns). Thus, alone and free in solution the area around the N-terminal R92C residue is the most flexible of the FM conjugates, near the C-terminal R239C residue is the least flexible, and a few residues before the αN have intermediate flexibility.

For the C-subunit-bound conjugates, parameter fitting was performed with the values of the slow correlation times ($\phi_{\text{slow}}$) fixed at 38 ns, the value predicted by the Hydropro computer program (version 7.0.2) (20) using the atomic coordinates of the Rlα(91–244) C-subunit complex (Protein Data Bank code 1U7E). Fixing $\phi_{\text{slow}}$ was performed to reduce the variability caused by the short lifetime of the reporter groups relative to the whole-body rotational correlation time (Table II). The fitting parameters generated by this procedure are summarized in Table II.

For all three conjugates, C-subunit binding was associated with relatively large increases in the amplitudes of the slower depolarization processes ($\beta_1$), which is consistent with a reduced range (angular excursion) of local (tether arm and/or adjacent α-carbonyl backbone) motions of the reporter groups. Specifically, C-subunit binding increased $\beta_1$ from 0.130 to 0.173 for FM-R92C, 0.154 to 0.241 for FM-T104C, and 0.173 to 0.215 for FM-R239C (Table II). The effects of C-subunit binding on the amplitudes ($\beta_2$) and rates ($\phi_{\text{fast}}$) of the fast decay processes were more complex. For the FM-R92C conjugate, C-subunit binding decreased the fast correlation time ($\phi_{\text{fast}}$) from 1.5 to 0.9 ns with no change in $\beta_1$, but an increase in the time 0 anisotropy ($\beta_1 + \beta_2$) from 0.219 to 0.259 (Table II), which is consistent with a restriction of sub-nanosecond local tether arm motions and faster local α-carbonyl backbone motions. For the FM-T104C conjugate, C-subunit binding increased $\phi_{\text{fast}}$ from 2.1 to 3.0 ns, decreased $\beta_1$ from 0.996 to 0.175, and increased time zero anisotropy from 0.251 to 0.281 (Table II), which is consistent with reduced motions of both α-carbonyl backbone and tether arm motions. For the FM-R239C conjugate, C-subunit binding...
decreased $\phi_{\text{fast}}$ from 2.4 to 1.7 ns and decreased $\beta_1$ from 0.081 to 0.052 without changing significantly the time zero anisotropy (Table II), suggesting a decrease in the range but not rate of adjacent $\alpha$-carbonyl backbone flexibility without constraining tether arm motions.

Effects of cAMP, (Rp)-cAMPS, and (S)p)-cAMPS on Time-resolved Anisotropy Decay of FM-Rlα (91–244) Conjugates—In the absence of C-subunit, the only ligand effects observed were with the reporter group attached to residue 239 near the end of $\alpha$C. Here, cAMP and (S)p)-cAMPS (and perhaps (Rp)-cAMPS as well) reduced the “fast” rotational correlation time ($\phi_{\text{fast}}$) from 2.4 to 1.6–1.7 ns and reduced the amplitude of the fast decay processes ($\beta_1$) from 0.081 to 0.65–0.68 (Table II). This would be consistent with an increased interaction of the end of $\alpha$C with the PBC and the protein core, which would reduce the range of motions. (Note that $\phi_{\text{fast}}$ is a complex function of both the range of motions and rate of diffusion, so a decrease in the range of motions will produce a decrease in $\phi_{\text{fast}}$.)

In studies of ligand effects on C-subunit-bound FM-Rlα (91–244) conjugates, C-subunit was added in 10-fold excess of the FM-Rlα (91–244) conjugates (0.4 $\mu$M) to minimize cAMP- or (S)p)-cAMP-induced dissociation. Under these conditions, the concentration of C-subunit was at least 50-fold greater than the $K_d$ for R-C binding in the presence of either cAMP or (S)p)-cAMPS (Table I), so no more than 1% dissociation would be expected to occur upon addition of either cAMP or (S)p)-cAMPS. For C-subunit-bound FM-conjugates, ligand-dependent effects were observed with the FM-T104C and the FM-R239C conjugates and here only with added cAMP and (S)p)-cAMPS. Specifically, for the reporter group attached to residue 104 near the C-terminal side of the linker domain, cAMP and (S)p)-cAMPS but not (Rp)-cAMPS reduced the amplitude of the slow decay processes ($\beta_2$) from 0.241 to 0.194–0.199, which was associated with a reduction of the time zero anisotropy from 0.280 to 0.230–0.240 without a significant change in $\phi_{\text{fast}}$ (Table II). This effect would be consistent with enhanced angular excursions of the local motions without significant changes in the rate of diffusion. For the FM-R239C conjugate, cAMP and (S)p)-cAMPS but not (Rp)-cAMPS increased the fast rotational correlation time ($\phi_{\text{fast}}$) from 1.7 to 2.3 ns without affecting the decay amplitudes (Table II). This effect would be consistent with enhanced constraints on the motions of the end of $\alpha$C due to increased interactions with the Rlα (91–244) core and/or C-subunit; the former is more likely. Together, these results suggest that cAMP and (S)p)-cAMPS decrease the interaction of the linker domain and $\alpha$C of Rlα with the C-subunit without affecting the interaction of the pseudosubstrate sequence with the catalytic cleft.

Effects of (Rp)-cAMPS on H/$^2$H Exchange in Rlα (91–244)- C-subunit Complexes—The actions of cAMP and the two phosphothioate analogs on the structure and dynamics of PKA were investigated further by amide H/$^2$H exchange analysis measured by ESI-Q-TOF mass spectrometry in combination with proteolytic fragmentation by pepsin as described under “Experimental Procedures.” In total, 21 proteolytic fragments from Rlα (91–244) that covered 84% of its primary sequence and 50 proteolytic fragments from the C-subunit that covered 85% of its primary sequence were identified. The extent of deuterium of the fragments from Rlα (91–244) and the C-subunit within 5 min is summarized in Tables III and IV. An example of the effects of deuteration and (Rp)-cAMPS on a fragment spanning the PBC (residues 204–221, m/z = 664.3) is illustrated in Fig. 5.

Plots of the time course of deuterium of fragments associated with significant (Rp)-cAMPS-induced changes in deuterium are illustrated in Figs. 6 and 7. The maximum number of deuterons exchanged during a 5-min deuterium oxide exposure was determined from fitting plots of the time course of deuterium to a single exponential equation. Results obtained by this method of data analysis are comparable with the results obtained by using the average number of deuterons exchanged following a 5-min incubation from three independent experiments (10, 21, 22).

Decreased exchange in the C-subunit was observed in two regions. The first region included the PBC spanned by five overlapping peptides (m/z = 1396.64, residues 188–201; m/z = 567.32, residues 202–212; m/z = 705.76, residues 202–221; m/z = 644.30, residues 204–221; and m/z = 500.83, residues 213–221) that showed a decrease in deuterium exchange (1–2 deuterons) (Table III). Subtractive analysis with these overlapping peptides enabled further localization of the site of decreased exchange upon (Rp)-cAMPS binding. For instance, the peptide 188–198 showed no difference in exchange, but the larger peptide 188–201 showed a protection of two deuterons upon (Rp)-cAMPS binding, indicating that residues 199–201 reflected (Rp)-cAMPS binding. Similarly, subtractive analysis of overlapping peptides (202–212, 204–212, 202–221, and 213–221) allowed narrowing down of the site of decreased exchange to residues 205–212 of the PBC. The decreased exchange seen in this region is consistent with the roles of Glu200 and Arg209 in coordinating the rare OH and phosphodiester moieties of cAMP, respectively (23). In the absence of any ligand, this region is highly shielded from solvent and/or structured because a large proportion of its residues do not exchange within 5 min (10). (Rp)-cAMPS binding into this pocket likely provides additional shielding, and this accounts for the observed decrease in deuterium exchange.

The second region of the R-subunit showing decreased exchange upon binding of (Rp)-cAMPS is a region spanning the critical Asp170 residue with three nested peptides spanning residues 157–172 (m/z = 846.89), 161–172 (m/z = 1322.58), and 162–172 (m/z = 1225.54). Subtractive analysis allowed narrowing down of the sites of decreased exchange of one deuteron to residues 162–172. One region showed increased exchange in amide exchange at the $\alpha$A helix (residues 136–143, m/z = 976.4) and is part of the peripheral interaction surface with the C-subunit (Table III).
For the C-subunit, (Rp)-cAMPS binding resulted in no difference in deuterium exchange (Table IV) in a majority (42 of 50) of all peptides analyzed. Seven peptides showed increased exchange at sites distal to the R-C intersubunit interface. Interestingly, a majority of the peptides showing increased exchange spanned regions of the protein outside the core kinase domain; these included the N-terminal αA helix and the C-terminal tail. Subtractive analysis of the five overlapping fragments that covered residues 237–267 localized a one-deuteron increase in residues 247–250 in αG and a one-deuteron increase in residues 251–261 in the loop connecting the αG and αH in the C-terminal strand. Thus, (Rp)-cAMPS induces limited unfolding and/or enhanced conformational flexibility that is spread almost globally through the C-subunit and, at least, a portion of Rlk(91–244) (Fig. 8).

We had hoped to analyze the effects of (S)p-cAMPS and cAMP on H/D exchange in holoenzyme complexes by performing the experiments under conditions that would minimize holoenzyme dissociation (high protein concentrations and an excess of either free C-subunit or Rlk(91–244)). Unfortunately, the presence of (S)p-cAMPS and excess subunit decreased the resolvability of the deuterated fragments and made meaningful analysis problematic, so the effects of (S)p-cAMPS and cAMP could not be determined.

**DISCUSSION**

The most significant result of our efforts to explore the actions of cAMP and two phosphorothioate analogs on the type Iα PKA holoenzyme is that (Rp)-cAMPS acts by increasing the affinity of Rlk(91–244) toward the C-subunit and thus is better described as an inverse agonist because it decreases the fractional dissociation of the holoenzyme and, in turn, its basal activity. Less exciting but still important is the finding that cAMP and its mimic appear to reduce the interaction of Rlk(91–244) with the C-subunit “contact” Sites 2 and 3 but probably not Site 1. Additionally, (Rp)-cAMPS binding to Rlk(91–244)C-subunit complex produces long range effects that propagate through both the R- and C-subunits to induce limited unfolding and/or enhanced conformational flexibility. To put these results in perspective, it is of value to review the conformational states of Rlk, the structural basis for R-C interaction, the major CAMP-dependent intramolecular signaling pathways that spread out from the PBC, and the physical consequences of sulfur for oxygen substitution into cAMP.

Rlk exists in two major conformations: one with high affinity toward the C-subunit and one with low affinity. The low affinity conformation is highly extended, almost barbell-shaped, with the two CNB domains at opposite ends of the molecule and linked by a combined, single αB and αC helix (αB/C). Cyclic AMP binding to the holoenzyme shifts the conformational equilibrium of Rlk from the high affinity to the low affinity conformation. Cyclic AMP binding to Rlk also reduces the
TABLE IV
(R$_p$)-cAMPS effects on amide H$^2$H exchange in the C-subunit complexed to Rlx(91–244)

### Non-overlapping 95% confidence intervals relative to control values were determined by the Prizm computer program.

| Fragment of C-subunit (m/z) (region) | Number of exchangeable amides | Charge (z) | Maximum amides exchanged (mean ± S.E.) | Control | Plus (R$_p$)-cAMPS |
|--------------------------------------|-------------------------------|------------|---------------------------------------|---------|------------------|
| 7–18 (700.32) (αA)                  | 11                            | 2          | 8.2 ± 0.05                            | 9.7 ± 0.19 |
| 14–31 (732.72) (αA)                 | 17                            | 3          | 4.3 ± 0.11                            | 5.3 ± 0.11 |
| 19–26 (461.25) (αA)                 | 7                             | 2          | 4.2 ± 0.10                            | 5.3 ± 0.37 |
| 19–27 (534.79) (αA)                 | 8                             | 2          | 4.4 ± 0.13                            | 4.9 ± 0.16 |
| 21–36 (953.5) (αA–β1)               | 14                            | 2          | 8.4 ± 0.19                            | 9.5 ± 0.22 |
| 27–40 (822.44) (αA–β1)              | 12                            | 2          | 7.7 ± 0.17                            | 8.3 ± 0.20 |
| 28–40 (765.90) (αA–β1)              | 11                            | 2          | 7.6 ± 0.12                            | 8.2 ± 0.19 |
| 42–55 (792.90) (β1)                 | 13                            | 2          | 1.9 ± 0.07                            | 2.0 ± 0.13 |
| 45–55 (597.83) (β1)                 | 10                            | 2          | 3.0 ± 0.11                            | 3.0 ± 0.14 |
| 60–71 (700.84) (β1–β2)              | 11                            | 2          | 0.4 ± 0.02                            | 0.9 ± 0.04 |
| 83–100 (727.74) (αB–αC)             | 17                            | 3          | 3.3 ± 0.09                            | 3.2 ± 0.13 |
| 92–100 (544.83) (αC)                | 8                             | 2          | 1.7 ± 0.06                            | 1.6 ± 0.09 |
| 98–103 (736.403) (αC)               | 4                             | 1          | 0.9 ± 0.02                            | 0.8 ± 0.04 |
| 98–104 (807.44) (αC)                | 5                             | 1          | 1.6 ± 0.03                            | 1.6 ± 0.09 |
| 104–108 (635.38) (β4)               | 4                             | 1          | Solvent-inaccessible                   | Solvent-inaccessible |
| 104–110 (869.48) (β4)               | 6                             | 1          | 1.0 ± 0.02                            | 1.2 ± 0.07 |
| 105–110 (770.41) (β4)               | 5                             | 1          | 1.0 ± 0.05                            | 1.1 ± 0.08 |
| 106–115 (1200.55) (β4)              | 9                             | 2          | 1.7 ± 0.06                            | 1.7 ± 0.11 |
| 109–116 (924.44) (β4)               | 7                             | 1          | 1.0 ± 0.02                            | 1.1 ± 0.06 |
| 119–126 (825.38) (β5)               | 7                             | 1          | 0.2 ± 0.02                            | 0.4 ± 0.02 |
| 122–128 (726.32) (β5–αD)            | 6                             | 1          | 1.0 ± 0.17                            | 0.8 ± 0.08 |
| 122–129 (855.36) (β5–αD)            | 7                             | 1          | 1.0 ± 0.14                            | 1.0 ± 0.29 |
| 133–145 (570.29) (αD–αE)            | 12                            | 3          | 1.2 ± 0.03                            | 1.3 ± 0.01 |
| 144–151 (470.26) (αE)               | 7                             | 2          | 3.0 ± 0.10                            | 2.9 ± 0.03 |
| 156–163 (989.49) (αE–F)             | 1                             | 0.3 ± 0.02  | 0.3 ± 0.01                            |
| 162–173 (743.94) (β6–67)            | 10                            | 2          | 0.5 ± 0.04                            | 0.3 ± 0.3  |
| 173–178 (673.35) (β7)               | 5                             | 1          | 1.3 ± 0.01                            | 0.9 ± 0.05 |
| 180–185 (722.36) (β8)               | 5                             | 1          | 0.3 ± 0.24                            | 0.2 ± 0.19 |
| 180–187 (926.46) (β8)               | 7                             | 1          | Solvent-inaccessible                   | Solvent-inaccessible |
| 182–187 (685.32) (β8)               | 5                             | 1          | Solvent-inaccessible                   | Solvent-inaccessible |
| 188–212 (981.52) (β9-activation loop) | 22                           | 3          | 3.5 ± 0.08                            | 3.5 ± 0.08 |
| 189–212(932.50) (β9-activation loop) | 21                           | 3          | 3.3 ± 0.08                            | 3.2 ± 0.09 |
| 198–211 (1418.73) (β9-activation loop) | 10                           | 1          | Solvent-inaccessible                   | Solvent-inaccessible |
| 205–211 (768.49)                    | 5                             | 1          | Solvent-inaccessible                   | Solvent-inaccessible |
| 222–227 (658.40) (αF)               | 5                             | 1          | Solvent-inaccessible                   | Solvent-inaccessible |
| 231–245 (826.90) (αF–αG)            | 11                            | 2          | 5.3 ± 0.12                            | 5.0 ± 0.19 |
| 232–239 (869.43) (αF)               | 5                             | 1          | 1.1 ± 0.03                            | 1.2 ± 0.02 |
| 241–247 (784.42) (αF–αG)            | 5                             | 1          | 3.0 ± 0.10                            | 2.9 ± 0.27 |
| 262–269 (890.48) (αH)               | 7                             | 1          | Solvent-inaccessible                   | Solvent-inaccessible |
| 262–273 (693.90) (αH)               | 11                            | 2          | Solvent-inaccessible                   | Solvent-inaccessible |
| 268–275 (741.50) (αH)               | 5                             | 1          | Solvent-inaccessible                   | Solvent-inaccessible |
| 268–274 (869.56) (αH)               | 6                             | 1          | Solvent-inaccessible                   | Solvent-inaccessible |
| 278–302(748.40) (αH–αI)             | 24                            | 4          | 7.0 ± 0.20                            | 8.1 ± 0.20 |
| 291–303 (831.41) (αH–αI)            | 12                            | 2          | 3.1 ± 0.16                            | 3.3 ± 0.22 |
| 298–303 (740.33) (αH–αI)            | 5                             | 1          | 2.4 ± 0.08                            | 2.6 ± 0.15 |
| 303–326 (689.87) (C-terminal tail)  | 19                            | 4          | 9.5 ± 0.12                            | 9.9 ± 0.20 |
| 303–327(706.64) (C-terminal tail)   | 20                            | 4          | 9.0 ± 0.17                            | 9.4 ± 0.17 |
| 305–327 (623.85) (C-terminal tail)  | 18                            | 4          | 10.0 ± 0.13                           | 10.5 ± 0.25 |
| 306–327 (632.34) (C-terminal tail)  | 17                            | 4          | 8.1 ± 0.06                            | 8.5 ± 0.13 |
| 335–346 (728.36) (C-terminal tail)  | 11                            | 2          | 7.0 ± 0.10                            | 7.9 ± 0.11 |
elements of the linker region (residues 98–120), the 3ıα loop (residues 133–140), the PBC (residues 198–211), and the extended αB plus αC helix (residues 226–244). The complementary interface of the C-subunit divides into three regions or sites. Site 1 includes elements of the glycine-rich loop (residues 50–55), αF (residues 218–233), and P + 1 loop (residues 198–205). Site 2 includes all or elements of the APE linker (residues 206–208), αF, and αG (residues 243–252). Site 3 includes elements of the activation loop (residues 193–197). Site 1 of the C-subunit interacts with the pseudosubstrate sequence of RIA (P – 3 to P + 1 residues); Site 2 interacts with the 3ıα loop, PBC, αB, and αC of RIA; and Site 3 interacts with elements of the RIA linker region and the extended αB plus αC helix.

Based on the x-ray and NMR structural analyses of isolated cAMP-bound and free RIA mutants, the effects of cAMP binding appear to be mediated through both direct and indirect intramolecular non-covalent bonding pathways that spread out from the PBC. Particularly noteworthy are the pathways from the PBC to αB and αC. The critical indirect pathway includes the relay point Asp170 at the N terminus of β3 that links to the equatorial phosphate oxygen of bound cAMP through Arg209 in the PBC to Arg226 at the N-terminal end of αB (7, 25). Substitution of Lys for Arg at residue 209 blocks binding to the CNB-A site (26) and transforms (Rp)-cAMPS into an agonist (27). A direct pathway connects the exocyclic equatorial phosphate oxygen of bound cAMP to the amide nitrogens of Leu201 and Gly199 in or next to the PBC. Additionally, secondary pathways link the PBC, αA, and αB. Specifically, αA and αB are linked through Asp146 and Arg230, whereas PBC and αB are linked through Leu203, Ile204, and Arg230. Mutational analysis (28) and inference from the x-ray structure of RIA(91–244) indicate a link between the 2'–OH of cAMP to Arg241 in αC via a Glu200 bridge in the PBC.

Functionally, the binding of cAMP to the PBC can be considered as a phosphorylation event that acts as an electrostatic switch. Arguably, the most important moiety of cAMP involved in the activation process is its phosphate oxygen in the equatorial position (Rp) anchored by a critical, conserved Arg209 (29). As discussed above, cAMP binding activates this electrostatic switch. Substitution of sulfur into the Rp position of cAMP yields an inverse agonist, whereas substitution of sulfur into the Sα position yields a cAMP mimic. In the case of (Rp)-cAMPS, the sulfur is bulkier than oxygen, and although the S–P bond is more electronegative than the O–P bond, the phosphorothioate sulfur is more polarizable, and its negative charge density is less than that of the corresponding phosphonate. The phosphorothioates are thus stronger acids and therefore less capable of hydrogen bonding (30–32). The combination of the larger size and reduced polarity of the S–P bond yields an entity that is unable to activate the Arg209–Asp170–Arg226 switch and stabilize RIA into a low affinity state toward the C-subunit.

The present fluorescence anisotropy results show that with RIA(91–244) C-subunit complexes cAMP and (Sαp)-cAMPS decrease the mobility of, at least, a portion of αC, presumably by activation of the above mentioned networks that would enhance the interactions of αB and αC with the PBC and the core of RIA (91–244). The anisotropy results also show that cAMP and (Sαp)-cAMPS increase the mobility of, at least, a portion of the linker region (around residue 104), which can be explained if there is a decrease in the interaction of the linker region with αC of RIA(91–244). From the crystal structure of the RIA(91–244) C-subunit complex (Protein Data Bank code 1U7E), this latter interaction is mediated by a non-covalent bond network connecting the linker region (via Glu101, Tyr103, Glu105, and Asp107) to αB and αC (via Asp227, Arg230, Arg231, and Met234). No evidence was found for cAMP or (Sαp)-cAMPS perturbation of the interaction of the pseudosubstrate sequence with C-subunit Site 1. Taken together, these results are consistent with cAMP and (Sαp)-cAMPS enhancing the interaction of αB and αC with the PBC and the core of RIA.
while decreasing the links between αB and αC with the linker region of RIX(91–244). All this leads to a reduction of the interaction of RIX with Sites 2 and 3 of the C-subunit probably without a direct effect on Site 1.

In the absence of C-subunit, the flexibility of the pseudosubstrate sequence and the linker regions, as indicated by the low electron densities of these regions in the x-ray crystal structures of cAMP-bound RIX(91–244), render intramolecular signaling pathways between the PBC and these regions unlikely. Highly flexible regions are poor conduits for intramolecular signaling because the dwell time of their conformational states is much shorter than the time

**FIG. 6.** Time course of deuterium incorporation into backbone amides in regions of RIX(91–244) subunit in C-RIX(91–244) in absence (○) and presence of (R<sub>p</sub>)-cAMPS (●) for peptides. A, m/z = 797.86, z = 2 (residues 136–148). B, m/z = 846.89, z = 2 (residues 157–172). C, m/z = 567.32, z = 2 (residues 202–212). D, m/z = 523.81, z = 2 (residues 230–238). The solid lines denote the best fit of the data to a single exponential equation. Error bars were calculated from 2 replicate experiments.

**FIG. 7.** Effects of (R<sub>p</sub>)-cAMPS on time course of deuterium incorporation into backbone amides in regions of C-subunit complexed to RIX(91–244). Data are plotted in the absence (○) and presence of (R<sub>p</sub>)-cAMPS (●) for the peptides. A, m/z = 700.32, z = 2 (residues 7–18). B, m/z = 732.72, z = 3 (residues 14–31); C, 673.35, z = 5 (173–178). D, m/z = 728.36, z = 2 (residues 335–346). The solid lines denote the best fit of the data to a single exponential equation. Error bars were calculated from 2 replicate experiments.
required to achieve the large scale conformational changes associated with functional states. The anisotropy decay results reported here also supports the flexible character of this region. Specifically, the amplitude of the slow decay processes ($\beta_2$; an inverse measure of the maximum diffusional excursion angle of the reporter group) is the smallest for FM-R92C (0.130; 38% of total decay) and somewhat larger for FM-T104C (0.154; 45% of total decay). Indeed, the fast rotational correlation time of FM-R92C is near the detection limit of our instrument ($\phi_{\text{fast}} = 0.9$ ns) and is consistent with rapid $\alpha$-carbonyl backbone motions. In this light, the failure to detect any effects of cAMP or its analogs on the N-terminal region of the pseudosubstrate or linker regions is not surprising. In the case of the end of $\alpha$C, both cAMP and (S$_p$)-cAMPS appeared to decrease both the amplitude ($\beta_i$) and the correlation time ($\phi_{\text{res}}$) of the fast anisotropy decay processes of the reporter group at residue 239, suggestive of a greater interaction of $\alpha$B and $\alpha$C with the core of Rl of Rl (91–244) upon cAMP or (S$_p$)-cAMPS binding.

Although the anisotropy measurements reveal little about the actions of (R$_p$)-cAMPS, results from H/$^2$H amide exchange analysis were more illuminating. (R$_p$)-cAMPS induces large conformational changes in the Rl (91–244)-C-subunit complex. In Rl (91–244), amide exchange increased in both $\alpha$A and $\alpha$C and decreased by one or two deuterons in the PBC, presumably through steric shielding effects of (R$_p$)-cAMPS binding to this site. It is important that the results confirm the importance of the Arg$^{209}$/Asp$^{170}$ relay in propagating the effects of cAMP binding to the R-C interface. This is one region that showed decreased exchange upon (R$_p$)-cAMPS binding and strongly suggests that the basis for the inability of (R$_p$)-cAMPS to facilitate dissociation of the C-subunit is the uncoupling of the charge relay linking Arg$^{209}$ and Asp$^{170}$ and is consistent with previous mutagenesis data (26, 29). In the C-subunit, changes in amide exchange occurred almost globally with single deuteron increases in fragments associated with the $\alpha$A-$\beta$1 loop, $\alpha$F, $\alpha$G-$\alpha$H loop, $\alpha$H-$\alpha$1 loop, and the C-terminal tail. Reduced amide exchange was observed in only two segments between residues 262 and 264 at the C-terminal end of $\alpha$H and residues 173–178, both distal to the R-C interface.

The changes are remarkable for at least two reasons. First, the near global increases in amide exchange were accompanied by a 5-fold increase in affinity of Rl (91–244) toward C-subunit, equivalent to a small change in relative free energy of binding ($\sim$0.9 kcal/mol). The increased amide exchange indicates increase solvent accessibility and/or conformational flexibility, which could be due to enhanced internal entropy and, in turn, provide an explanation for the increased affinity. (An enhancement in enthalpy would primarily produce stabilization.) It must be pointed out that some of the regions that showed increased exchange are within the N terminus of the C-subunit and span helix $\alpha$A and the C terminus. The $\alpha$A in PKA is very critical for thermostability and more importantly for proper orientation of the two subdomains to maintain the substrate-binding cleft (33). The increased exchange in this region and other regions distal to the intersubunit interface corresponding to one to two additional deuteron exchanged potentially reflects the increased conformational mobility of both lobes of the C-subunit. A similar effect has been seen in p38 mitogen-activated protein kinase where the dephosphorylated protein shows increased exchange throughout the molecule, reflecting increased relative conformational mobility of the unphosphorylated protein (34). The enhanced mobility is likely responsible for enhanced binding to the R-subunit upon binding of (R$_p$)-cAMPS. Also, one needs to consider that (R$_p$)-cAMPS shifts slightly the equilibrium from a state with low affinity toward the C-subunit to one with high affinity. Second, the fact that these near global conformational changes occur in the absence of the activation of the electrostatic phosphate oxygen switch discussed above point to the importance of other components of cAMP such as the 2’-OH and 3’-O of the ribose and/or the adenine ring to produce long range conformational changes albeit with limited functional significance.

It should be noted that Gesellchen et al. (35) previously reported that (R$_p$)-cAMPS increased the affinity of Rl to toward C-subunit using a non-quantitative bioluminescence resonance energy transfer assay. Here, we have put numbers to this effect and have described (R$_p$)-cAMPS more accurately as an inverse agonist.
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This article contains supplemental Fig. 9.

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