Arginine 210 Is Not a Critical Residue for the Allosteric Interactions Mediated by Binding of Cyclic AMP to Site A of Regulatory (RIα) Subunit of Cyclic AMP-dependent Protein Kinase*

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The guanidinium groups of conserved arginines in the two intrachain cAMP-binding sites of regulatory (R) subunit of cAMP-dependent protein kinase have been implicated in the allosteric interactions by which cAMP binding leads to kinase activation. We have investigated the functional role of Arg-210, the conserved arginine in site A of murine type I R subunit, by analyzing the effects of nine different substitutions at this residue on cAMP binding and allosteric properties of bacterially expressed RIα subunits. All substitutions reduced the cAMP binding affinity of site A, but the magnitude of reduction varied from several hundredfold to 10^6-fold. The differential effects of the different substitutions could not easily be rationalized by interactions with cAMP and might, in part, reflect interactions with other residues in the unoccupied cAMP-binding pocket. None of the Arg-210 substitutions appeared to disrupt the allosteric interaction by which occupation of site A slows dissociation of cAMP from site B, although the effect was difficult to elicit in full with mutations that had strong effects on cAMP binding. The two weakest substitutions, Arg-210 → Ile and Arg-210 → Thr, could be shown to have essentially no effect on the allosteric interaction by which occupation of site A reduces the affinity of R subunit for the catalytic subunit. The weaker mutations had a smaller effect on kinase activation by the suboptimal activator Rβ-adenosine cyclic 3',5'-phosphorothioate than by cAMP, suggesting that the analog largely bypasses interactions with the guanidinium group of Arg-210.

Activation of cAMP-dependent protein kinase results from allosteric interactions by which cAMP binding to two intrachain cAMP-binding sites (sites A and B) in kinase regulatory (R) subunit decreases by 5 orders of magnitude or more the affinity of R for the catalytic (C) subunit (reviewed in Ref. 1). Studies with analogs of cAMP implicate the ribose-cyclic phosphate moiety of cAMP in the activation process (2, 3). The adenine ring is thought to contribute to the specificity and high affinity of cAMP binding (2, 4). A recent crystal structure of a large fragment containing the cAMP-binding domains of R subunit corroborated predictions of an earlier model based on the structure of the catalobite activator protein of Escherichia coli that most of the important contact residues for interaction with the ribose-cyclic phosphate of cAMP are in β-strands 6 and 7 of an antiparallel β-roll structure forming one face of the cAMP-binding pockets (5, 6). It appears from this structure that Glu-201 in site A and Glu-325 in site B interact with the 2'-hydroxyl group of cAMP and that the guanidinium groups of Arg-210 in site A and Arg-334 in site B interact with the equatorial exocyclic oxygen of the cAMP phosphate group (5). Mutations at these residues or at the conserved Gly residues immediately upstream of the conserved Gly residues markedly reduced the affinities of the mutated sites for cAMP (7–10). Arg-242, in the long α-helix perpendicular to the β-roll structure for site A, appears to contribute to the stability of the site A-binding pocket by electrostatic interaction with Glu-201 (5) and is essential for allosteric interaction between sites A and B (11).

Rβ-phosphorothioate (Rβ-cAMPS) and dithioate analogs of cAMP have been described as antagonists of wild-type kinase (12–14). Actually, early studies suggested that Rβ-cAMPS was a partial agonist for cAMP-dependent protein kinase (15, 16), and this view was reinforced by a more recent report showing that Rβ-cAMPS activates wild-type kinase in the absence of ATP or enzymes with mutant R subunits (Arg-210 → Lys or Ala-98 → Ser) in the presence of ATP (17). The apparent importance of the equatorial exocyclic oxygen of cAMP (missing in Rβ-cAMPS) for full kinase activation led to the suggestion that interaction between this oxygen and a positively charged amino acid side chain was responsible for the cAMP-dependent conformational change in the R subunit underlying the C subunit release (15). From the structure of the cAMP-binding sites,

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†† The abbreviations used are: R subunit, regulatory subunit of cAMP-dependent protein kinase; C subunit, catalytic subunit of cAMP-dependent protein kinase; Rβ, or Sβ-cAMPS, Rα, or Sα-adenosine cyclic 3’,5’-phosphorothioate; Bz-cAMP, Nβ-benzoyl-cAMP; MOPS, 3-(N-morpholino)propanesulfonic acid; bis-tris propane, 1,3-bis[(tris(hydroxymethyl)methylamino)propane; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

Because the mouse RIα subunit used for these studies has one more amino acid in its amino-terminal domain than does the porcine homolog used by some other investigators, the residue numbers used in this report are higher by one than those used in some other reports (e.g. Refs. 5, 7, 17, and 18).
the affinity of R for C subunit.

To better evaluate the role of Arg-210 in cAMP binding and allosteric functions of site A, we introduced a variety of amino acid substitutions at this site in bacterially expressed recombinant murine Rα subunit. Ile-210, Ser-210, and Thr-210 mutations had been identified as spontaneous or mutagen-induced substitutions found in K, mutant isolates of S49 mouse lymphoma cells (19) and were transferred from mutant cDNAs to the bacterial expression plasmid. Asn, Asp, Gln, Glu, His, and Lys substitutions were generated by site-directed mutagenesis to assess the role of ionic and/or hydrogen-bonding interactions in the functions of Arg-210. Although the mutations all reduced the binding affinity of site A, the affinities of the mutant sites varied over a range of more than 1,000-fold. Using R subunits with the mutations that least affected cAMP binding, we found that the guanidinium group of Arg-210 is unnecessary for either the allosteric effect of cyclic nucleotide binding that couples the kinetic properties of site B with occupation of site A or the allosteric coupling by which occupation of site A decreases the affinity of R for C subunit.

EXPERIMENTAL PROCEDURES

Materials

Chemicals and Biochemicals—2,8-3HICAMP (30 C/mmol), [γ-32P]-ATP (3000 C/mmol), and Aquassure scintillation mixture were from DuPont NEN; the phosphate acceptor heptapeptide Leu-Arg-Ala-Ser-Leu-Gly (Kemptide) was synthesized and high performance liquid chromatography-purified by the Molecular Biology Resource Facility of the University of Oklahoma Health Sciences Center; N'-benzoyl-cAMP (Bz-cAMP), gMP, ATP, MOPS, and bis-tris propane were from Sigma; R, cAMP was purified further by high performance liquid chromatography to give concentrations of 0.2–0.4 μM in dissociation buffer containing 0.5 mM 2-mercaptoethanol, and 0.1 mM EDTA. Urea solution was prepared freshly as for the standard kinase assay (above). In the experiment of Fig. 7, 50 mM MOPS (pH 7.0) was used as the buffer in place of bis-tris propane.

Results

Initial characterization of mutants with substitutions at Arg-210 suggested that site A function was compromised, but affinities of the mutant sites could not be estimated with our standard filter-kinase assay. (11, 21). For the subunit reassociation experiments of Figs. 2, 6, and 10 μl each of R and C subunits at three times their final concentrations were mixed in reconstitution buffer containing ATP (as the magnesium salt) at the concentrations to be used in subsequent kinase assays (30 μM for Fig. 5 and 0.2 mM for Figs. 2, 6, and 7). For the experiments of Figs. 2 and 7, R and C subunits were reconstituted in a similar manner at 50 times their final concentration and then diluted to three times the final concentration immediately before use. After preincubations as described in figure legends, either 10 or 20 μl of reconstituted subunits were mixed with reconstitution buffer containing 0.5 μCi of [γ-32P]-ATP to prime the reaction mixture to give a final concentration of 0.2 mM for the experiments of Figs. 2, 6, and 7 and 0.1 mM for that of Fig. 5. For the activation experiments of Figs. 2 and 7, cAMP or cAMP analogs were included in the labeling mixture to give the final concentrations indicated. 30-μl reaction volumes were preincubated at 30°C and samples were assayed for kinase activity after a 20°C incubation of R subunit results solely from the interaction of cAMP with site A and represents the sum of the results from the alternative assay based on the finding that cAMP-dependent quenching of the endogenous Trp fluorescence of R subunit results solely from the interaction of cAMP with site A (11, 25). The R subunit concentrations (0.2–0.4 μM) were apparently near or above the dissociation constants (Kd values) for cAMP binding to site A of the Ile-210 and Thr-210 mutants, but, for the other R subunit preparations, the Trp fluorescence quenching could be titrated with increasing concentrations of cAMP to yield apparent Kd values. In terms of relative effects on site A affinity, the mutations could be or- dered as follows: Thr, Ile < Tyr < Asn < His. The relative effects on site A and site C affinities were estimated with increasing concentrations of cAMP to yield apparent Kd values. In terms of relative effects on site A affinity, the mutations could be or- dered as follows: Thr, Ile < Tyr < Asn < His. The relative effects on site A and site C affinities were estimated with increasing concentrations of cAMP to yield apparent Kd values. In terms of relative effects on site A affinity, the mutations could be or- dered as follows: Thr, Ile < Tyr < Asn < His. The relative effects on site A and site C affinities were estimated with increasing concentrations of cAMP to yield apparent Kd values. In terms of relative effects on site A affinity, the mutations could be or- dered as follows: Thr, Ile < Tyr < Asn < His. The relative effects on site A and site C affinities were estimated with increasing concentrations of cAMP to yield apparent Kd values. In terms of relative effects on site A affinity, the mutations could be or- dered as follows: Thr, Ile < Tyr < Asn < His. The relative effects on site A and site C affinities were estimated with increasing concentrations of cAMP to yield apparent Kd values. In terms of relative effects on site A affinity, the mutations could be or- dered as follows: Thr, Ile < Tyr < Asn < His. The relative effects on site A and site C affinities were estimated with increasing concentrations of cAMP to yield apparent Kd values.
Despite numerous attempts to determine site A affinities for the Ile-210 and Thr-210 mutants by [3H]cAMP-binding assays, we were ultimately unsuccessful. The rapid off-rates of the mutant sites compromised retention of labeled nucleotide in the standard filter-binding assay, and data from equilibrium dialysis experiments were ambiguous at best. In attempts to estimate more accurately the effects of the Ile-210 and Thr-210 mutations on site A binding, we combined the position 210 substitutions at Arg-210 by incubating these double mutant R subunits with Gly-324 to Asp mutation, which inactivates site B (10, 11). Fig. 2 shows activation curves of kinase reconstituted by incubating these double mutant R subunits with purified C subunit. Fig. 2A compares the effects of the Ile-210/Asp-324 and Lys-210/Asp-324 mutations with that of the Asp-324 mutation alone on activation by Bz-cAMP. For this experiment, we used relatively low concentrations of R and C subunits to facilitate activation of enzyme with the Lys-210 activation constant (K_{a}), and the effect of the Asp-324 mutation alone. Equilibrium dialysis experiments were ambiguous at best. In attempts to join the data points with smooth curves.

The curves in this and all other figures are free-hand attempts to join the data points with smooth curves.

Fig. 2. The effects of Ile-210, Lys-210, and Thr-210 mutations on the Bz-cAMP-mediated activation of holoenzymes with a mutationally inactivated site B (Gly-324 → Asp). R subunits with the Asp-324 mutation alone (●), Ile-210 and Asp-324 (■), Lys-210 and Asp-324 (▲), or Thr-210 and Asp-324 mutations (▼) were preincubated with C subunit for 1 h at room temperature in the presence of ATP as described under “Experimental Procedures.” Kemptide, [γ-32P]ATP, and Bz-cAMP were then added, and the samples were incubated for 5 min at 30 °C before processing to measure phosphate transfer. The concentration of C subunit in assays was 0.5 nM, and the concentrations of R subunit were 1 nM (A) or 10 nM (B). For this figure and Figs. 4 and 7, bound cyclic nucleotide is an insignificant proportion of total, so the total concentrations shown are free concentrations.

and Thr-210 mutations having about an order of magnitude less effect on cAMP-binding affinity than the Lys-210 mutation.

Figs. 3 and 4 present evidence that mutant R subunits with substitutions at Arg-210 retain the allosteric interaction by which occupation of site A retards dissociation of cAMP from site B (9). Fig. 3 shows results of experiments in which cAMP concentrations were varied in low (Fig. 1A) and high salt (Fig. 1B) to titrate the effect of site A on dissociation of prebound [3H]cAMP from site B. All of the mutant preparations exhibited a cAMP-dependent slowing of site B dissociation, and this suggested that the mutant A sites were able to couple allosterically with intrachain B sites. In terms of dose-response behavior, the apparent order of the various mutations was consistent with the data of Figs. 1 and 2, with Thr-210 and Ile-210 the weakest, Gln-210 and Glu-210 strongest, and the remainder more or less bunched in between. The dose-response curves in high salt were steeper than those in high salt. (Shallower titration curves were also observed when Trp quenching was assayed in high salt (data not shown).) High salt shifted the dose-response curves to the right by varying amounts, but the

3 M. M. Symcox and R. A. Steinberg, unpublished observations.
slower dissociation of $[^3H]cAMP$ from site B in high salt made it easier to detect unambiguously the effects of high cAMP on site B dissociation from R subunits with the strongest (Gln-210 and Glu-210) mutations. For wild-type and all the mutant R subunits, site A affinities estimated by the assays of intrachain coupling were substantially lower than those determined by more direct assays of cAMP binding (above).

The apparent discrepancies between cAMP concentrations required to saturate site A (Figs. 1 and 2) and those required to slow cAMP dissociation from site B (Fig. 3) suggested that something more than simple occupation of site A might be required for the allosteric effect on site B. Furthermore, it was unclear from the experiments of Fig. 3 whether or not the stronger mutations disrupted partially the allosteric coupling between sites A and B. In an attempt to understand better how intrachain coupling was related to occupation of site A, we repeated the coupling experiments using either Bz-cAMP or cGMP as the ligand for site A. Bz-cAMP binds to site A with an affinity of R for C subunit, we again used R subunits with a double mutant R subunit all retained high affinity binding for cGMP as the ligand for site A, with Kemptide and $[^3H]cAMP$ bound to site B of mutant R subunits. Dissociation of $[^3H]cAMP$ bound to Ile-210 ($\bullet$), Lys-210 ($\square$, ■), or Gln-210 R subunits ($\triangle$, ▲) was measured in the presence of various concentrations of Bz-cAMP (□, ■, ▲), or cGMP (●, ■, ▲) in high salt as for the experiment of Fig. 3B. Small symbols and dotted lines reproduce the data for cAMP from Fig. 3.

FIG. 3. The effect of cAMP binding to site A on dissociation of $[^3H]cAMP$ from site B in R subunits with mutations at Arg-210. Dissociation of $[^3H]cAMP$ bound to wild-type or mutant R subunits was assayed in the absence or presence of competing unlabeled cAMP to give the total C subunit required for the activity assay is above the apparent $K_a$ for the interaction between wild-type R and C subunits, this

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assay does not give true $K_d$ values but is sensitive to changes in association kinetics. All three R subunit preparations inhibited the C subunit with similar dose responses, suggesting that the basal interaction between R and C subunits was not significantly impaired by the mutations at Arg-210.

Fig. 6 shows the results of similar titration experiments performed in the presence of high levels of Bz-cAMP. For these experiments the preincubation and assay times were extended so that the kinase reactions proceeded, for the most part, after the subunits had reached their equilibrium states of association. All three concentrations of Bz-cAMP had similar effects on the interaction of the Asp-324 mutant R subunit with the C subunit, suggesting that the wild-type site A in this subunit remained saturated with the cyclic nucleotide at concentrations at or above 0.6 mM (data for only 15 mM Bz-cAMP is shown). In contrast interaction of the double mutant R subunits with C subunit was weakened progressively as the cyclic nucleotide was increased from 0.6 to 15 mM. At 15 mM Bz-cAMP, the apparent affinity of the Ile-210/Asp-324 R subunit for C subunit was only slightly higher.

Because the experiments of Fig. 6 suggested that the guanidinium group of Arg-210 was not essential for the coupling between site A and the C subunit-binding site, we decided to reinvestigate the activation potential of $R_p$-cAMPS. Fig. 7 compares activation profiles for kinase reconstituted with wild-type or mutant R subunits using either cAMP (Fig. 7A) or $R_p$-cAMPS (Fig. 7B) as an activator. Because the analog slowly hydrolyzes to cAMP during storage, we used a preparation that had been freshly repurified by high performance liquid chromatography; in other experiments we pretreated the analog with cAMP phosphodiesterase and obtained qualitatively similar results. Under the conditions of these assays, cAMP gave essentially full activation with the wild-type, Thr-210, Lys-210, and His-210 enzymes and partial activation with the Gln-210 enzyme. The apparent $K_a$ values for all the mutant enzymes were more than 10-fold higher than that for wild-type kinase. Since activation in the mutant enzymes was largely through site B, the differences in apparent $K_a$ values were small, but the rank order for all but the Gln-210 mutant was consistent with the binding data of Fig. 1.

FIG. 6. The effect of Ile-210 and Thr-210 mutations on the cyclic nucleotide-mediated reduction in affinity of R for C subunit. R and C subunits were mixed in the presence of ATP and Bz-cAMP as described under "Experimental Procedures" and incubated for 1 h at room temperature. Kemptide and [$\gamma$-32P]ATP were then added, and the samples were incubated for an additional hour at 30°C before processing to measure phosphate transfer. C subunit was at 0.33 nM in the reactions, and the final concentrations of Bz-cAMP were 0.6 (●), 3.0 (■), or 15 mM (▲, △). Filled symbols show data for Ile-210/Asp-324 (A), or Thr-210/Asp-324 mutant R subunits (B), and open triangles (dotted curves) show data for the Asp-324 singly mutant R subunit.

FIG. 7. Activation of wild-type, Thr-210, Lys-210, His-210, and Gln-210 holoenzymes by either cAMP or $R_p$-cAMPS. R and C subunits were reconstituted overnight at 4°C at 75 times their final concentrations as described under "Experimental Procedures." For assay, the reconstituted mixtures were diluted 25-fold, and 10-µl portions were added to 20 µl of mixture containing Kemptide, [$\gamma$-32P]ATP, and cyclic nucleotide. Final concentrations of R and C subunits were 20 and 3.3 nM, respectively, and incubations were for 15 min at 30°C. R subunits were wild-type (●) or had Thr-210 (□), Lys-210 (▲), His-210 (■), or Gln-210 mutations (△). An additional set of incubations contained C subunit alone (○).
subunit in which site A was inactivated by a Glu-200 mutation. The poor activation of these mutant enzymes by the analog suggested that the activation observed with wild-type or the other mutant enzymes required the function of site A as well as of site B.

DISCUSSION

The cAMP-occupied site A-binding pocket is a compact structure stabilized by both intrachain interactions and interactions with the bound cAMP (5). The ribose phosphate moiety of cAMP is apparently anchored by interactions of its 2'-hydroxyl group with the γ-carboxyl group of Glu-201 and the α-amino group of Gly-199, and of its 5'-terminal cyclic oxygen with the guanidinium group of Arg-210 and the α-amino group of Ala-203. The guanidinium group of Arg-210 has additional interactions with the backbone carbonyls of Asn-172 and Gly-200 and the β-carboxyl group of Asp-171 to stabilize the binding pocket and, perhaps, transmit signals to adjacent regions. In view of the importance of the guanidinium group for these interactions involving Arg-210, we were surprised to find that Ile or Thr substitutions for Arg-210 were relatively benign in their effects on cAMP binding (Figs. 1 and 2). Ile at position 210 had less effect on site A function than the basic amino acids Lys or His, suggesting that hydrophobic interactions involving the stem of Arg-210 might be important for cAMP binding. These could be either direct interactions with cAMP or, more likely, intrachain interactions stabilizing the cAMP-bound conformation of the binding pocket. The importance of such hydrophobic interactions is also supported by the tighter binding of cAMP to site A of the Thr-210 mutant R subunit than to that of the Ser-210 mutant protein. Substitution of the negatively charged Glu for Arg-210 decreased site A affinity by almost 10^6-fold, but this effect could not be attributed simply to electrostatic repulsion of cAMP; Asp had an effect that was weaker by nearly 2 orders of magnitude than that of Glu, and Gln had an effect that was stronger than that of Asp. The stronger effects of Glu-210 and Gln-210 mutations than those of Asp-210 and Asn-210 mutations suggest either steric interference between these larger amino acids and cAMP or novel hydrogen-bonding interactions involving the side groups of these amino acids that stabilize the unoccupied conformation of the site A cAMP-binding pocket.

In a previous study we showed that allosteric interaction between sites A and B required Arg-242 and suggested that this interaction involved communication through the 2'-hydroxyl group of cAMP and Glu-201 (11). The site A structure shows clearly that Glu-210 and Arg-242 are positioned to effect this interaction and suggests further, that Trp-261 is also involved (5). Consistent with this view of coupling between sites A and B proceeding from interactions at the 2'-hydroxyl group of cAMP, the mutations at Arg-210 did not obliterate A-B coupling, and a full coupling response was observed with the Ile-210 and Thr-210 mutant subunits (Fig. 3). The effects of the various residue 210 substitutions on the cAMP-mediated reduction of site B off-rate were related to their effects on site A affinity, but not in a simple linear fashion. Only the Ile-210 and Thr-210 mutants exhibited wild-type levels of coupling with cAMP, and the Lys-210 mutant could be fully coupled with Bz-cAMP (Figs. 3 and 4). For the more severely impaired mutants and the Lys-210 mutant with cAMP or cGMP, the shallow dose-response curves left it unclear whether or not even saturating levels of cyclic nucleotide would elicit coupling to wild-type levels. Wild-type R subunit cAMP concentrations effective for coupling (65 μM) and used fluorescence polarization to monitor pressure-induced dissociation. We found a Kd at atmospheric pressure of about 1 nm in the absence of ATP, and this was shifted to about 10 μM by adding ATP (M. M. Symcox and R. A. Steinberg, unpublished results). An affinity of about 50 μM was estimated by Døskeland et al. (1) from considerations of cyclic nucleotide and ATP-binding constants.

4 To measure basal R-C affinities, we reconstituted holoenzyme from a 2,5-dansyl-labeled C subunit and an amino-terminally truncated R subunit (that could not dimerize, but interacted normally with C subunit) and used fluorescence polarization to monitor pressure-induced dissociation. We found a Kd at atmospheric pressure of about 1 nm in the absence of ATP, and this was shifted to about 10 μM by adding ATP (M. M. Symcox and R. A. Steinberg, unpublished results). An affinity of about 50 μM was estimated by Døskeland et al. (1) from considerations of cyclic nucleotide and ATP-binding constants.
presence of ATP the \( S_p \), but not the \( R_p \), stereoisomer of cAMP was an effective agonist. Both isomers were agonists in the absence of ATP. Holoenzyme reconstituted with a Lys-210 mutant R subunit could be activated by \( R_p \)-cAMPs in both the presence and absence of ATP (17). In our experiments, rather than facilitating activation by \( R_p \)-cAMPs, the Lys-210 mutation partially suppressed activation with the analog (Fig. 7). Wild-type holoenzyme could be partially activated by \( R_p \)-cAMPs in the presence of ATP with the level of activation dependent on the experimental conditions, particularly the concentrations of kinase subunits (Fig. 7, and data not shown).

Holoenzymes formed with wild-type or mutant R subunits with the weak Ile-210 or Thr-210 substitutions were activated to similar levels by \( R_p \)-cAMPs, and the Lys-210 mutant enzyme was activated somewhat less (Fig. 7, and data not shown). Holoenzymes with the stronger site A mutations His-210 and Gln-210 exhibited even less activation by \( R_p \)-cAMPs, thus demonstrating the importance of site A function for activation by this analog. While the apparent activation constants for cAMP were about 10-fold higher for Ile-210 and Thr-210 mutant enzymes than for the wild-type species, the difference in apparent activation constants with \( R_p \)-cAMPs for Ile-210 and Thr-210 mutant enzymes was about 10-fold higher for Ile-210 and Thr-210 mutant enzymes than for the wild-type species, the difference in apparent activation constants with \( R_p \)-cAMPs as activator was only about 2–3-fold. This suggests either that the guanidinium group of Arg-210 is more important for binding of cAMP than of \( R_p \)-cAMPs or that the guanidinium group actually interferes with binding of \( R_p \)-cAMPs. The partial agonist activity of \( R_p \)-cAMPs reported in two early studies (15, 16) has been attributed to contamination of the analog with cAMP (12). Our experiments with the analog was purified as described for the pure preparations of previous reports (e.g. Ref. 12), and the differences in relative resistances of wild-type and mutant enzymes to activation by cAMP and \( R_p \)-cAMPs argues against the possibility that the activation seen with the analog resulted from contaminating cAMP. We suspect that our detecting partial agonist activity with the wild-type enzyme results from the combination of relatively low enzyme concentration and high Km for wild-type enzymes in our experiments when compared with those that detected only antagonist activity (e.g. Refs. 12 and 17).

Our results indicate that the guanidinium group of Arg-210, while important for high affinity binding of cAMP to site A, plays little if any role in the allosteric responses provoked by cAMP binding to site A. As discussed above, it is not surprising that Arg-210 is unnecessary for the kinetic coupling of site B dissociation with site A occupation. On the other hand, that substitution of Ile or Thr for Arg-210 had little or no effect on the allosteric response by which cyclic nucleotide binding to site A reduces the affinity of R for C subunit was quite unexpected. It would appear possible that it is the interactions of the exocyclic oxygens of cAMP phosphate with the backbone amides of Ala-203 and Ala-211 (5) rather than with the guanidinium group of Arg-210 that are important for allosteric transmission. The sulfur substituent in \( R_p \)-cAMPs reduces the binding affinity to site A of R subunit by almost 800-fold, where the axial substitution in \( S_p \)-cAMPs reduces this affinity by only about 5-fold (28), consistent with steric interference between the guanidinium group of Arg-210 and the equatorial sulfur. The suboptimal activation potential of the \( R_p \)-cAMP analog may result from both the inability of its reduced binding energy to maintain the R subunit conformation with lowest affinity for C subunit and the failure of its equatorial sulfur substituent to interact effectively with the backbone amide of Ala-203.

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