Identifying the Molecular Switches That Determine Whether (R_p)-cAMPS Functions as an Antagonist or an Agonist in the Activation of cAMP-Dependent Protein Kinase I†

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ABSTRACT: Previous investigations revealed that under physiological conditions in the presence of MgATP the phosphorothioate analogue of cAMP, (R_p)-cAMPS, is a competitive inhibitor and antagonist for cAMP for cAMP-dependent protein kinases I and II [DeWit et al., (1984) *Eur. J. Biochem. 142*, 255-260]. For the type I holoenzyme, the antagonist properties of (R_p)-cAMPS are shown here to be absolutely dependent on MgATP. In the absence of MgATP, (R_p)-cAMPS serves as a weak agonist with a K_a of 7.9 μM. The high-affinity binding of MgATP imposes a barrier on cAMP-induced activation of the homoenzyme—a barrier that both cAMP and (S_p)-cAMPS, but not (R_p)-cAMPS, can overcome. In the absence of MgATP, this barrier no longer exists, and (R_p)-cAMPS functions as an agonist. The holoenzyme also was formed with mutant regulatory subunits. Replacing the essential arginine, predicted to bind the exocyclic oxygens of cAMP, in site A with lysine abolishes high-affinity binding of cAMP to site A. The holoenzyme formed with this mutant R-subunit is activated by (R_p)-cAMPS in both the presence and absence of MgATP. These results suggest that the stereospecific requirements for holoenzyme activation involve this guanidinium side chain. Mutations that eliminate the high-affinity binding of MgATP, such as the introduction of an autophosphorylation site in the autoinhibitory domain, also generate a holoenzyme that can be activated by (R_p)-cAMPS. In the case of the type II holoenzyme, (R_p)-cAMPS is an antagonist in both the presence and absence of MgATP, emphasizing distinct roles for MgATP in these two forms of cAMP-dependent protein kinase.

The holoenzyme form of cAMP-dependent protein kinase (cAPK) is dissociated and, as a consequence, activated by the cooperative binding of cAMP to the regulatory (R) subunit, thus releasing the active monomeric form of the catalytic (C) subunit [for reviews, see Beebe and Corbin (1986) and Taylor et al. (1990)].

Each regulatory subunit monomer has a well-defined domain structure that includes a dimer interaction site at the amino-terminus followed by a substrate-like autoinhibitor site and two tandem cAMP binding sites, A and B, at the carboxy-terminus. Although these two cAMP binding sites show extensive sequence similarities (Takio et al., 1984; Titani et al., 1984), they, nevertheless, differ with respect to their rate kinetics for binding cAMP (Øgreid & Døskeland, 1983) and their specificity for analogues of cAMP (DeWit et al., 1984; Øgreid et al., 1989). Many cAMP analogues have been synthesized in an effort to better understand the specificity requirements for kinase activation and ultimately to identify antagonists having the potential to block kinase activation (Jastorff, 1979; Revankar & Robins, 1982). To date, these analogues have provided considerable information about the requirements for specificity and have served to discriminate between the two cAMP-binding sites (Øgreid et al., 1989). However, only one class of cAMP analogues (Figure 1) have proved to be effective as antagonists: the chiral phosphothioate analogue (R_p)-cAMPS its adenine-base modified derivatives (Dostmann, 1987; Genieser et al., 1988) and the double sulfur analogue $cAMPS_2$ (Parker-Botelho et al., 1988). (R_p)-cAMPS binds competitively to both cAMP-binding sites and prevents the dissociation of the holoenzyme (Rothermel & Botelho, 1988). In contrast, (S_p)-cAMPS functions as an agonist of cAMP by binding to both sites and causing the complex to dissociate.

Cyclic AMP binding to the holoenzyme shows positive cooperativity, with Hill coefficients typically in the range of 1.5-1.6. The primary cooperativity is between site A and site B in a single protomer (Øgreid et al., 1985; Ringheim & Taylor, 1990). Based on kinetic evidence, the best model indicates that cAMP-binding site B is more accessible in the holoenzyme than site A, and binding of cAMP to site B then induces a conformational change that makes site A more accessible (Øgreid & Døskeland, 1981). According to this model, binding to site A is the key event that causes dissociation of the wild-type holoenzyme.

Several mutant forms of the regulatory subunit of cAMP-dependent protein kinase have been isolated by phenotypic screening (Øgreid et al., 1988; Steinberg et al.,1987) and by using recombinant techniques (Bubis et al., 1988a,b). Many of these mutants contain single amino acid changes in the cAMP-binding sites that drastically reduce the affinity for cAMP. For example, replacing the essential Arg in site A, Arg209, with Lys is sufficient to abolish high-affinity binding of cAMP to site A. This R-subunit forms the holoenzyme extremely well and in the absence of MgATP can be activated by cAMP binding only to site B.

The studies described here were designed to ask whether the holoenzyme formed with a mutant R-subunit that is defective in site A is still inhibited by (R_p)-cAMPS or whether inhibition by (R_p)-cAMPS requires a functional cAMP-binding site A. The results indicate that site A is important for inhibition with (R_p)-cAMPS but, in addition, demonstrate that the inhibition of the native cAMP-dependent protein kinase I by (R_p)-cAMPS shows an absolute requirement for

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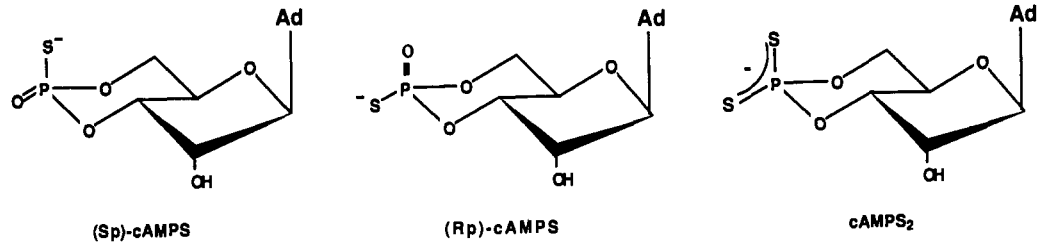

FIGURE 1: Phosphorothioate analogues of cAMP.

MgATP. In the presence of MgATP, (R_p)-cAMPS is an antagonist of cAMP, whereas in the absence of MgATP it is an agonist.

Experimental Procedures

Materials. Reagents were obtained from the following companies: (R_p)- and (S_p)-adenosine cyclic 3',5'-phosphorothioate [(R_p)/(S_p)-cAMPS] were purchased from BioLog, Bremen; cAMP and MES were from United States Biochemical Corp.; methanol and acetonitrile (HPLC grade) were from Fisher Scientific; and lactate dehydrogenase (LDH), pyruvate kinase, and bovine heart phosphodiesterase (PDE) were from Boehringer Mannheim. The peptide used in the spectrophotometric assay was synthesized on an Applied Biosystems peptide synthesizer at the Peptide and Oligonucleotide Facility at the University of California, San Diego. All other reagents were of analytical grade.

Adenosine Cyclic 3',5'-Phosphorothioates. The purity of the R_p and S_p diastereomers of adenosine cyclic 3',5'-phosphorothioate was analyzed by high-performance liquid chromatography (HPLC) using a reverse-phase C-18 column (Vydac). (S_p)-cAMPS was free of cAMP and contained <0.2% of the R_p diastereomer. (R_p)-cAMPS, however, was contaminated with 0.3% cAMP. Removal of cAMP was achieved by treating the compound with phosphodiesterase, since the enzyme cannot hydrolyze (R_p)-cAMPS (Van Haastert et al., 1984). The incubation contained 10 mM (R_p)-cAMPS, 10 mM Tris-HCl, pH 7.6, 2 mM $MgCl_2$, and 300 mg of PDE/mL (Van Haastert et al., 1987). After 1 h at 30 °C, the reaction was stopped by boiling the sample for 1 min. A control experiment showed that the untreated (R_p)-cAMPS sample activated the holoenzyme type I at high concentrations (>50 μM), commensurate with the low levels of cAMP contamination that were observed. In contrast, the PDE-treated sample did not activate up to an analogue concentration of 1 mM. Before use, (R_p)- and (S_p)-cAMPS were checked routinely by HPLC to verify that no contaminating cAMP was present.

Native and Recombinant Proteins. Porcine C-subunit was purified from porcine heart according to Nelson and Taylor (1981). Recombinant murine C-subunit was obtained as described by Slice and Taylor (1989). The recombinant type I regulatory subunit was purified by ion-exchange chromatography (Saraswat et al., 1988). The type II regulatory subunit was isolated from porcine heart (Zoller et al., 1979). The mutant type I regulatory subunits, R209K and A97S, were obtained as described previously (Bubis et al., 1988a,b; Durgerian & Taylor, 1989).

Holoenzyme Formation. The holoenzyme was formed by dialyzing the R-subunit with a 10% excess of C-subunit under conditions described previously (Bubis et al., 1988a,b). Reconstitution of cAPK(II) was complete following dialysis for 24 h at 0 °C, independent of MgATP. Reconstitution of cAPK(I) in the presence of MgATP was also complete after 24 h of dialysis, while reconstitution in the absence of MgATP required more extensive dialysis (>5 days). After holoenzyme formation was complete, CM-Sepharose CL-6B was used to remove the excess C-subunit. Holoenzyme formation was confirmed by visualizing the protein bands following polyacrylamide gel electrophoresis and by assaying for catalytic activity in the absence and presence of saturating (10 μM) amounts of cAMP. cAPK(I) formed in the presence of MgATP could be stripped of bound nucleotide by dialysis against buffer containing 2 mM EDTA for at least 48 h. Both recombinant and mammalian C-subunits were used for holoenzyme formation, and the corresponding holoenzymes gave identical results.

Holoenzyme Activation. Protein kinase activity was assayed by the coupled spectrophotometric method of Cook et al. (1982). Assays were carried out at room temperature, and the fraction of maximal C-subunit activity present was determined as a function of cAMP or the cAMP analogues. The synthetic peptide L-R-R-N-S-I was used as substrate. In a typical experiment, 350 μL of 25 nM holoenzyme was preincubated for 20 min at room temperature with various concentrations of cyclic nucleotide. This incubation is subsequently referred to as the preincubation mix. In this way it was possible to establish the effect that the presence or absence of MgATP has on the dissociation of holoenzyme before the reaction mixture is added to the actual assay solution, which obviously contains MgATP. To measure the activity, 300-μL aliquots of the preincubation mix were combined with 700 μL of the assay solution, and the decrease in NADH absorption followed for at least 2 min. All assays were carried out in duplicate. The reproducibility was typically ±5%. Each experiment was carried out independently at least twice. In all cases, the specific activity corresponding to maximum (100%) activation was comparable.

Results

Mutant Holoenzyme (R-Subunit: R209K). Replacing Arg209 with Lys in site A of the type I regulatory subunit is sufficient to abolish high-affinity cAMP binding to site A but leaves site B relatively intact (Bubis et al., 1988a,b). In the absence of MgATP, holoenzyme formed with this mutant enzyme can be activated readily by cAMP binding with a high affinity to the nonmutated site B (Bubis et al., 1988a,b). The capacity of the two phosphorothioate analogues of cAMP, (S_p)-cAMPS, and (R_p)-cAMPS, to activate this mutant holoenzyme containing a defective site A was tested to determine whether (R_p)-cAMPS, in particular, functions as an antagonist as it does for the wild-type holoenzyme. As seen in Figure 2A, (R_p)-cAMPS is not an antagonist for this mutant holoenzyme. Instead it activates with a K_a of 22.4 μM. The K_a's for activation by cAMP and (S_p)-cAMPS are 130 nM and 3.8 μM, respectively, as summarized in Table I.

This mutant holoenzyme also was shown previously to be very sensitive to MgATP. MgATP binds with a high affinity to the type I holoenzyme serving as a "lock" to make the holoenzyme more resistant to activation by cAMP (Hofmann et al., 1975; Neitzel et al., 1991). The effect of MgATP on activation of this mutant holoenzyme is even more pronounced.

Table 1: Activation Constants (K_a) for Holoenzymes with cAMP and Phosphorothioate Analogues of cAMP[a]

	cAMP		(S_p)-cAMPS		(R_p)-cAMPS	
R_2C_2	+Mg/ATP	-Mg/ATP	+Mg/ATP	-Mg/ATP	+Mg/ATP	-Mg/ATP
WT I	98 nM (1.30 ± 0.07)	26 nM (1.58 ± 0.22)	650 nM (1.23 ± 0.12)	56 nM (1.26 ± 0.08)	no act.	7.9 μM (0.80 ● 0.10)
R209K	6.4 μM (1.37 ● 0.10)	130 nM (0.73 ± 0.19)	70 μM (1.83 ± 0.13)	3.8 μM (0.6)	244 μM (1.15 ± 0.20)	22.4 μM (0.82)
A97S	52 nM (2.05 ● 0.22)	81 nM (1.35 ± 0.08)	320 nM (1.39 ± 0.17)	215 nM (0.76 ± 0.04)	69 μM (1.19 ± 0.11)	36 μM (0.83 ± 0.04)
WT II	540 nM (1.43 ● 0.11)	655 nM (2.08 ± 0.13)	2 μM (1.24 ± 0.11)	2.9 μM (0.95 ± 0.07)	no act.	no act.

[a] Hill coefficients are given in parentheses.

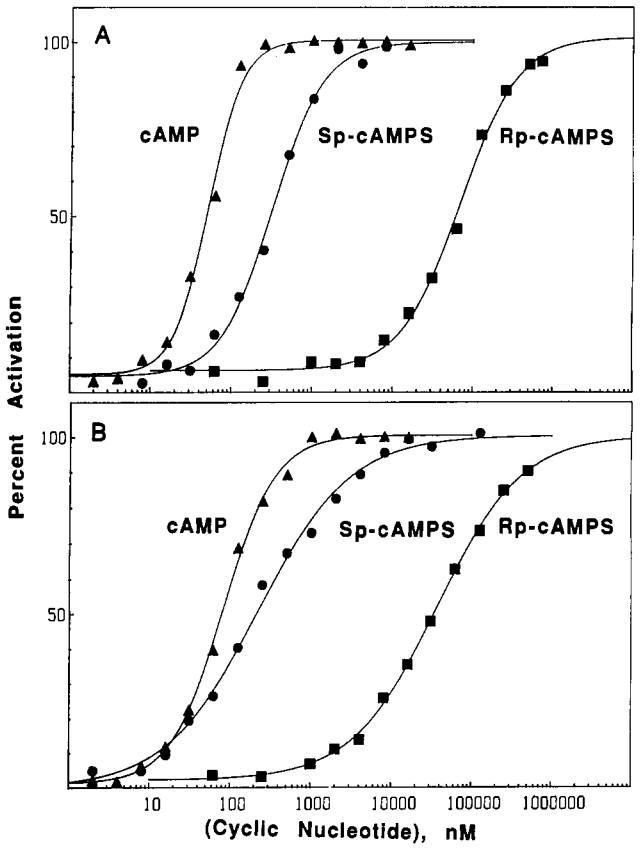

FIGURE 2: Activation of the mutant holoenzyme with cAMP and phosphorothioates of cAMP analogues. The holoenzyme (20–30 nM) was prepared with a mutant form of the R-subunit where Arg209 was replaced with Lys. Panel A shows activation when the preincubation was carried out in the absence of MgATP with cAMP (●), (S_p)-cAMPS (▲), and (R_p)-cAMPS (■). Panel B shows activation of the holoenzyme when preincubation with the cyclic nucleotides was carried out in the presence of MgATP.

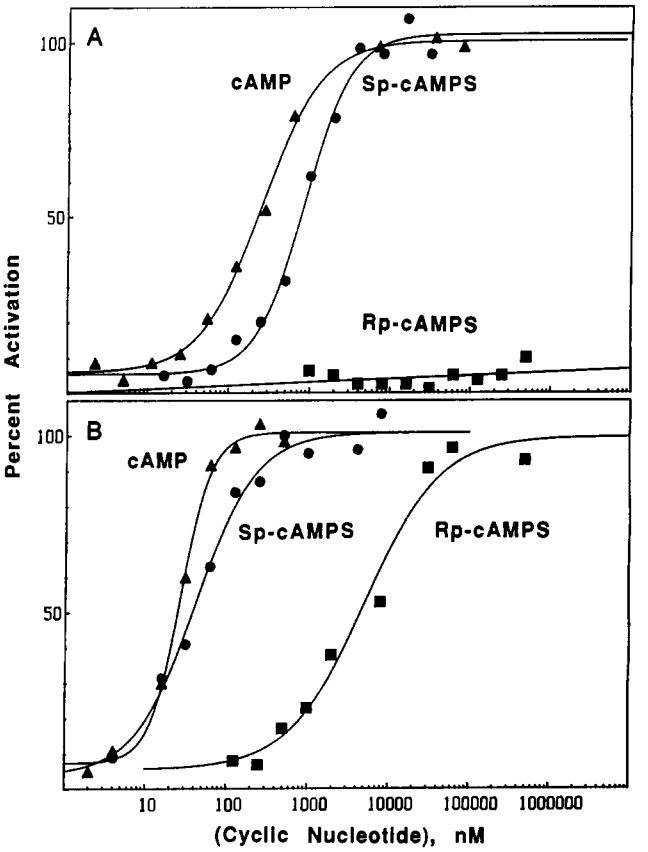

FIGURE 3: Activation of type I holoenzyme with cAMP and cAMPS analogues. Type I holoenzyme activation is shown in the presence of MgATP (panel A) and absence of MgATP (panel B) with cAMP (●), (S_p)-cAMPS (▲), and (R_p)-cAMPS (■). The percent activation was plotted against increasing concentrations (2 nM to 500 μM) of cyclic nucleotide. Holoenzymes were preincubated with the cyclic nucleotide both in the presence and absence of MgATP prior to the spectrophotometric assay.

The mutant holoenzyme still binds MgATP with a high affinity (10 nM), but the K_a (cAMP) for activation is greater than 1 μM. This represents a 50-fold increase in K_a in contrast to the 5-fold increase in K_a for the wild-type holoenzyme in the presence of MgATP. Given the pronounced effect of MgATP on holoenzyme activation, the effects of (S_p)-cAMPS and (R_p)-cAMPS were tested in the presence of MgATP. As seen in Figure 2B, even though this mutant holoenzyme is very resistant to activation by cAMP in the presence of MgATP, it can nevertheless be activated by (R_p)-cAMPS. Thus, for this mutant holoenzyme, (R_p)-cAMPS cannot function as an antagonist under any conditions, either in the presence or absence of MgATP.

Type I Holoenzyme. Previous studies demonstrating the antagonistic properties of (R_p)-cAMPS for the type I holoenzyme were consistently carried out in the presence of MgATP. In light of the above results, these earlier studies were repeated under two conditions: one where preincubation of the holoenzyme with the cyclic nucleotide was carried out in the absence of MgATP, and the other in the presence of MgATP. As indicated in Figure 3A, when type I holoenzyme was preincubated with cAMP, (S_p)-cAMPS, and (R_p)-cAMPS in the presence of MgATP, the activation was similar to what was observed previously when the holoenzyme was added directly to the assay solution containing cyclic nucleotide. For both cAMP and (S_p)-cAMPS, activation showed similar positive cooperativity, with the analogue having an 6–7-fold greater K_a (Table I). As reported previously, (R_p)-cAMPS did not activate the holoenzyme over the entire concentration range from 2 nM to 500 μM. Although binding per se is not shown here, Bothello et al. (1988) established unambiguously

that (R_p)-cAMPS binds to both sites but does not cause the complex to dissociate. The lack of activation is not due to lack of binding.

In contrast, when the preincubation was carried out in the absence of MgATP, the results shown in Figure 3B were observed. Under these conditions, (R_p)-cAMPS no longer functioned as an antagonist but instead served as a weak agonist with an K_a of 7.9 μM. When the preincubation was carried out in the absence of MgATP, the S_p diastereomer still activated the holoenzyme; however, the K_a did not differ significantly from cAMP (Table I). As a consequence, MgATP not only severely interferes with the capacity of (R_p)-cAMPS to activate the holoenzyme but also magnifies the distinction between cAMP and (S_p)-cAMPS.

The cooperativity for cAMP activation was slightly enhanced with a Hill coefficient 1.6 when the preincubation was carried out in the absence of MgATP, compared to 1.3 in the presence of MgATP, consistent with earlier reports showing the effect of MgATP on cAMP binding (Hofmann et al., 1975). The cooperativity was not altered by the presence or absence of MgATP when (S_p)-cAMPS was used to dissociate the holoenzyme. Under both conditions the Hill coefficient was approximately 1.2. Activation by (R_p)-cAMPS in the absence of MgATP, with a Hill coefficient of 0.8, showed no positive cooperativity.

Type II Holoenzyme. The type II holoenzyme does not bind MgATP with a high affinity but instead is autophosphorylated at the hinge region of the R^{II}-subunit in the presence of MgATP. To determine whether autophosphorylation alters the antagonistic properties of (R_p)-cAMPS, the type II holoenzyme was also incubated with (R_p)-cAMPS, both in the presence and absence of MgATP. Preincubation of the R^{II}-subunit alone with MgATP leads to stoichiometric phosphorylation at Ser95. In the absence of MgATP, the R^{II}-subunit is primarily (>80%) in its dephosphorylated state, on the basis of SDS–gel electrophoresis (see inset of Figure 4A). Its conversion to the phosphorylated form is presumably due to trace contamination of the purified R^{II}-subunit with C-subunit, which is almost impossible to avoid.

As seen in Figure 4A,B, activation constants for cAMP and (S_p)-cAMPS were both actually decreased when the preincubation was carried out in the presence of MgATP. This decrease in the K_a for activation by cAMP following phosphorylation was observed previously (Hofmann et al., 1975) and is in contrast to the type I holoenzyme where MgATP increases the K_a for cAMP activation. For the type II holoenzyme (R_p)-cAMPS did not activate the holoenzyme, either in the absence or presence of MgATP. Like cAPK(I), the Hill coefficient for cAMP activation was increased in the absence of MgATP. The activation of cAPK(II) with (S_p)-cAMPS in the absence of MgATP showed no cooperativity with a Hill coefficient of 0.95.

Effect of Mutations in the Hinge Region of the R^I-Subunit. In addition to mutations in the cAMP-binding sites, point mutations have been introduced into the autoinhibitor region of the R^I-subunit. One such mutation replaced Ala97 with Ser thereby generating an autophosphorylation site in the R^I-subunit. Like the R^{II}-subunit, this mutant R^I-subunit can be autophosphorylated in the holoenzyme complex; dissociation of the subunits is not required (Durgerian & Taylor, 1989). This holoenzyme also does not bind MgATP with a high affinity, presumably because the γ-phosphate of ATP is transferred immediately to the R-subunit as is the case for the type II R-subunit. The results shown in Figure 5 and summarized in Table I demonstrate that the holoenzyme formed with this mutant R-subunit also can be activated by (R_p)-cAMPS and that activation is independent of MgATP.

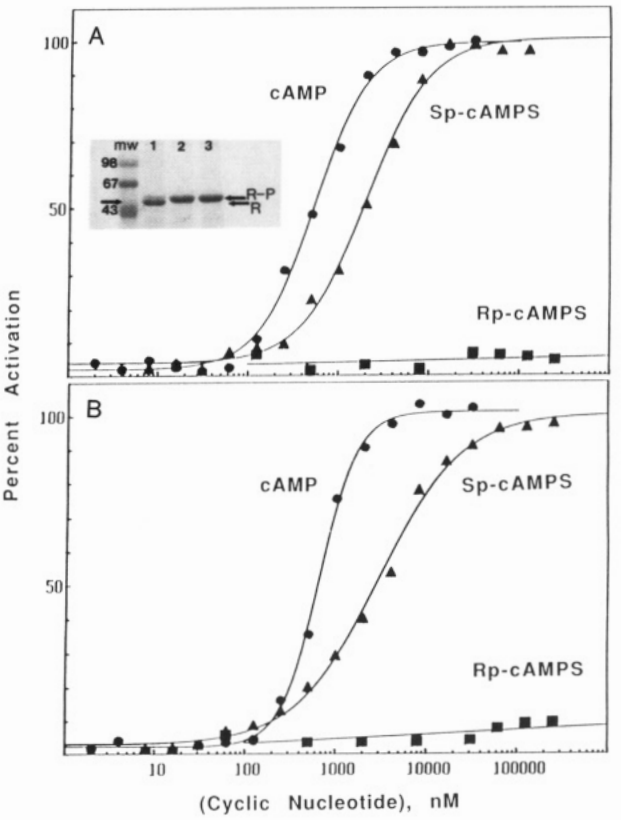

FIGURE 4: Activation of type II holoenzyme with cAMP and cAMPS analogues. Type II holoenzyme was preincubated with cyclic nucleotide in the presence (panel A) and absence (panel B) of MgATP. Symbols are as indicated in Figure 1. The inset shows the phosphorylation state of the purified type II regulatory subunit. The purified R^{II}-subunit was subjected to SDS–polyacrylamide gel electrophoresis run according to Laemmli (1970). Protein bands were visualized by staining with Coomassie blue. Arrows indicate the mobilities of the phosphorylated and dephosphorylated forms of the R-subunit. Lane 1 represents the purified R-subunit. Lane 2 is the same protein following incubation for 1 h with 0.5 mM MgATP. Lane 3 was incubated with 0.5 mM MgATP and a 1% molar ratio of C-subunit. The lane at the far left shows molecular weight markers.

Discussion

The R-subunit of cAPK exists in at least two stable conformational states in vivo. These two conformations can be designated as the cAMP-bound conformation (B-form), where all four cAMP-binding sites in the dimer are occupied with cAMP, or the holoenzyme conformation (H-form), where the cAMP-free R-subunit dimer exists as a stable complex with two C-subunits. Ultimately, we want to understand the molecular events that cause the regulatory subunit to switch from one conformation to the other. In the case of cAPK(I), MgATP binds to the the H-form with a high affinity (10 nM), and, hence, in the cell cAPK(I) will exist almost exclusively as a complex with MgATP as indicated below (Hofmann et al., 1975). The binding of MgATP increases the K_a for activation by approximately 5–10-fold with MgATP thus serving as a "lock" that stabilizes the holoenzyme complex. In order to understand the type I cAMP-dependent protein kinase, one must consider both nucleotides, cAMP and MgATP.

$$\underset{\text{H-form}}{R_2C_2\cdot MgATP_2} + 4cAMP \rightleftharpoons \underset{\text{B-form}}{R_2(cAMP)_4} + 2C\cdot MgATP$$

The high-affinity MgATP-binding site is one of the important features distinguishing the type I and type II holoenzymes. Both the R^I- and the R^{II}-subunits contain an au-

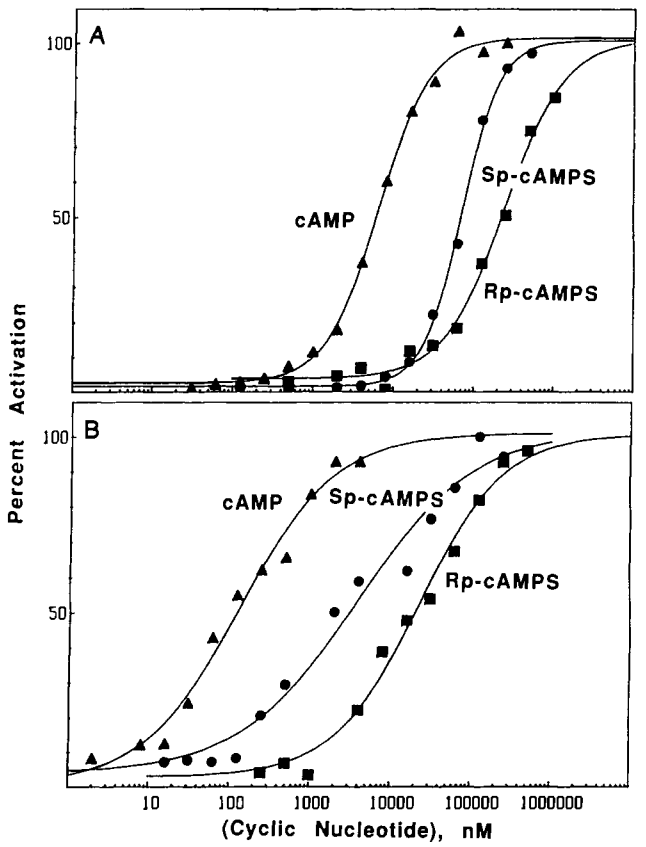

FIGURE 5: Activation of the holoenzyme formed with R-subunit containing a mutation at the autoinhibitory site, A97S. In panel A incubation was carried out in the absence of MgATP, while in panel B MgATP was included in the incubation with cyclic nucleotide prior to assaying. Symbols are as indicated in Figure 1.

toinhibitor site approximately 90–100 residues from the amino-terminus. In the case of the R^{II}-subunits, this site contains an autophosphorylation site, Arg-Arg-Val-Ser, and this Ser can be autophosphorylated readily in the holoenzyme complex (Rosen & Erlichman, 1975; Takio et al., 1982). In contrast, the type I R-subunit contains a pseudophosphorylation site, with the Ser typically replaced by Gly or Ala (Titani et al., 1984). MgATP binds to the type I holoenzyme complex but no suitable group is available to accept the phosphate. Replacement of this Ala with Ser generates and R^{I}-subunit that can be readily autophosphorylated by an intramolecular process (Durgerian & Taylor, 1989). Thus, in the holoenzyme, the autoinhibitor site of the R-subunit occupies the peptide-binding site of the C-subunit, preventing access of other substrates. On the basis of analogue studies, the ATP-binding site in the type I holoenzyme shares the same adenine-binding region as the free C-subunit; however, the environment surrounding the ribose ring and the β,γ-phosphates changes (Hoppe et al., 1977, 1978). These differences presumably account for the three order of magnitude increase in affinity for MgATP when free C-subunit and holoenzyme are compared.

Considerably more is known about the cAMP-binding sites, particularly the cAMP-bound (B) conformation, since the R-subunits share extensive sequence similarities with the catabolite gene activator protein (CAP) in *E. coli*. On the basis of the crystal structure of CAP, a model was proposed for each cAMP-binding domain (Weber et al., 1987). The general features of this site include (1) an invariant arginine that in CAP binds to one exocyclic oxygen of the phosphate moiety, (2) an invariant glutamic acid that in CAP hydrogen bonds to the 2'-OH of cAMP, and (3) interaction of the inward-facing surface of a long α-helix (C-helix) with the adenine ring of cAMP. The exocyclic oxygens of cAMP are important for recognition by the R-subunit on the basis of the early analogue studies (Jastorff et al., 1979); however, it was the CAP model that that linked recognition of the exocyclic oxygens with two essential arginine side chains, Arg209 and Arg333 in site A and site B, respectively. In the regulatory subunits the guanidinium group of each essential arginine is predicted to chelate through two or more bonds to cAMP instead of through the single bond that is observed for CAP (Dostmann et al., 1990). At this point little is known regarding the specific contacts that these invariant residues make in the H-form of the regulatory subunit since the only crystal structure available is that of CAP with bound cAMP.

With regard to the H-form of the R^{I}-subunit, let us consider first the native R^{I}-subunit and then the mutant R209K. In the case of the holoenzyme formed with the native R^{I}-subunit, the antagonist properties of (R_p)-cAMPS depend absolutely on the presence of MgATP. In the absence of MgATP, (R_p)-cAMPS is an agonist. For the type I holoenzyme, it is the presence of MgATP that imposes the stringent stereospecific features for cAMP activation.

Let us now consider the holoenzyme formed with the mutant R-subunit, R209K and, specifically, whether the stereospecific features of the phosphorothioate analogues require a functional site A. This mutant, in general, emphasizes the importance of steric interactions involving the guanidinium group of Arg209 both in the H-form and in the B-form of the regulatory subunit. When the Arg is replaced with Lys, a positive charge is conserved; however, any potential for stereospecificity in either conformation is lost. As in the case of the holenzyme formed with the native regulatory subunit, the stereospecificity does not seem to be critical in the absence of MgATP since binding of cAMP, as well as both (S_p)-cAMPS and (R_p)-cAMPS, to site B is sufficient to dissociate the holoenzyme complex. Thus, in either case, whether size A is occupied as in the case of the native holoenzyme or not as in the case of the mutant holoenzyme, (R_p)-cAMPS can dissociate the complex when MgATP is absent. This mutant holoenzyme does retain a high-affinity binding site for MgATP, and, in fact, in the presence of MgATP it becomes extremely resistant to activation by cAMP—much more resistant than the native holoenzyme. Nevertheless, in the presence of MgATP the mutant holoenzyme is activated by (R_p)-cAMPS when under the same conditions the native holoenzyme is not. Replacement of Arg209 with Lys abolishes the requirements for stereospecificity and under no conditions can (R_p)-cAMPS function as an antagonist. The precise role of this guanidinium side chain in the holoenzyme is unknown; however, these results suggest that the guanidinium side chain of Arg209 will play an equally important role in stabilizing the ATP-bound form of the holoenzyme complex.

While a high-resolution crystal structure obviously is required in order to fully understand the molecular events that lead to the interconversion of the H- and B-forms of the regulatory subunit, the model proposed in Figure 6 begins to deliniate the stepwise events that are involved in this process and to explain the stereospecific features of this conformational change. First, it is clear that the molecular switch that converts the H-form of the R^{I}-subunit into the B-form must involve several components. At one end is the γ-phosphate of ATP in close proximity to the hinge region of the regulatory subunit. At the other end of this switch must be the interaction of cAMP with the essential Arg in site A, Arg209. According to the model proposed in Figure 6, the side chain of Arg209

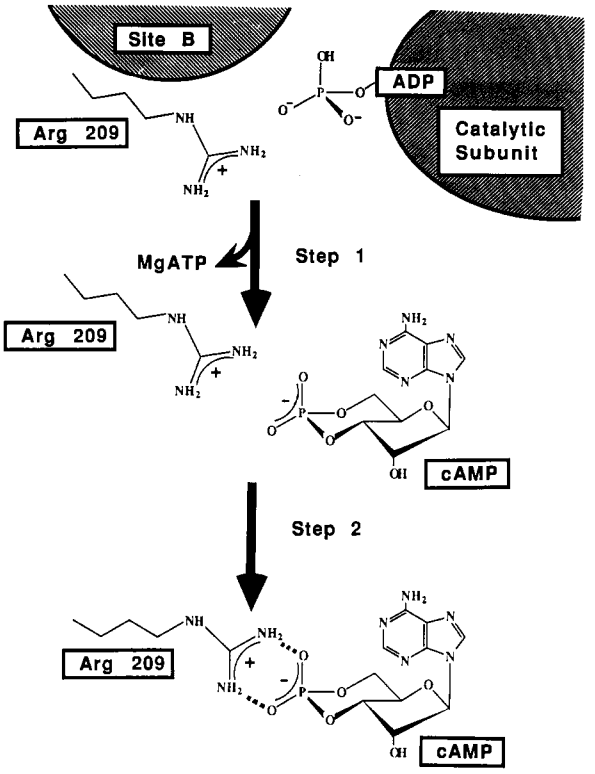

FIGURE 6: Model showing the stepwise activation of the type I holoenzyme.

in the H-form is influenced by at least two molecular signals: one from MgATP and one from the B-domain. Following cAMP binding to domain B, it is only the MgATP signal that remains. The subsequent conversion from the H-conformation to the B-conformation is a two-step process. The first step is to break the barrier imposed by MgATP. This must involve either direct or indirect competition for the side chain of Arg209, and it is this step where stereospecificity is critical. Only the S_p-isomer can accomplish this step. The second step in this conformational change is the allignment of the guanidinium side chain with the exocyclic oxygens of cAMP following the displacement of MgATP. In the absence of MgATP, only step 2 is required, and the only distinction between the stereoisomers (R_p)-cAMPS and (S_p)-cAMPS is in the K_a: both are agonists. Therefore, it is the first step, the displacement of MgATP, that the R_p-isomer cannot accomplish either because of steric hindrance due to the larger sulfur group or because of an inability to assume the required ionic configuration.

In the mutant protein, replacing Arg209 with Lys conserves a single positive charge but any opportunity for stereospecificity in either the H-form or the B-form is lost. In addition, because the charge is perhaps further from the cAMP due to the shorter Lys side chain and because it can only form one ionic interaction instead of two, it is more difficult to activate when MgATP is bound. When MgATP is not bound, however, the altered side chain is not capable of maintaining the holoenzyme conformation when site B is occupied. Mutations in the hinge region also can eliminate the high-affinity binding of MgATP, and in these mutants also only step 2 is required for holoenzyme dissociation.

MgATP exerts a different effect on the type II holoenzyme. Binding of MgATP results in the immediate transfer of the γ-phosphate to Ser95 at the hinge region. The phosphorylated form of cAPK(II) is slightly more sensitive to cAMP activation (Figure 2), and reassociation of R- and C-subunits is facilitated by dephosphorylation (Rangel-Aldao & Rosen, 1977). Clearly the phosphate at Ser95 of the R^{II}-subunit is not serving the same role with regard to cAMP-binding as the γ-phosphate of MgATP bound to the cAPK(I). For cAPK(II), the antagonistic properties of (R_p)-cAMPS are independent of autophosphorylation. These results thus emphasize further the differences between the two holoenzymes.

Registry No. (*Rp*)-cAMPS, 73208-40-9; (*Sp*)-cAMPS, 71774-13-5; cAMP, 60-92-4; MgATP, 1476-84-2; Ala, 56-41-7; Arg, 74-79-3; protein kinase, 9026-43-1.

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