Role of MgATP in the Activation and Reassociation of cAMP-Dependent Protein Kinase I: Consequences of Replacing the Essential Arginine in cAMP Binding Site A†

James J. Neitzel,‡ Wolfgang R. G. Dostmann, and Susan S. Taylor*
Department of Chemistry, University of California, San Diego, La Jolla, California 92093
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ABSTRACT: The type I form of cAMP-dependent protein kinase binds MgATP with a high affinity, and binding of MgATP decreases the affinity of the holoenzyme for cAMP [Hofmann et al. (1975) *J. Biol. Chem. 250*, 7795]. Holoenzyme was formed here with a mutant form of the bovine recombinant type I regulatory subunit where the essential arginine in site A, Arg-209, was replaced with Lys. Although this mutation does not significantly change the high-affinity binding of MgATP to the holoenzyme, it does abolish high-affinity binding of cAMP to site A. In the absence of MgATP, binding of cAMP to site B is sufficient to promote dissociation of the holoenzyme complex and activation of the catalytic subunit [Bubis et al. (1988) *J. Biol. Chem. 263*, 9668]. In the presence of MgATP, however, holoenzyme formed with this mutant regulatory subunit is very resistant to cAMP. The K_d(cAMP) was greater than 1 μM, and the K_a(cAMP) increased 60-fold from 130 nM to 6.5 μM in the presence of MgATP. Thus, MgATP serves as a lock that selectively stabilizes the holoenzyme and inhibits activation. Both site A and site B are shielded from cAMP in the presence of MgATP. These results suggest that Arg-209 may play a role in stabilizing the MgATP·holoenzyme complex in addition to its role in binding the exocyclic oxygens of cAMP when cAMP is bound to the regulatory subunit. The catalytic subunit also reassociates rapidly with this mutant regulatory subunit, and in contrast to the wild-type regulatory subunit, holoenzyme formation does not require MgATP.

cAMP-dependent protein kinase is a tetrameric enzyme composed of two regulatory (R) and two catalytic (C) subunits. Following binding of 4 mol of cAMP, this inactive holoenzyme complex dissociates into two monomeric catalytic subunits and a regulatory subunit dimer (R_2). The free catalytic subunit, the active form of the enzyme, transfers the phosphate of ATP to specific serine and threonine residues on protein or peptide substrates [for reviews, see Beebe and Corbin (1986), Bramson et al. (1984), and Taylor et al. (1990)]. The regulatory subunit of cAMP-dependent protein kinase is a dimensionally asymmetric dimer with a frictional coefficient of 1.4–1.5 (Zoller et al., 1979) whose primary known function is to bind to the catalytic subunit in the absence of cAMP and maintain the enzyme in an inactive state. Several unique forms of regulatory subunit exist and are generally categorized as type I and II based on the order in which they elute from an anion-exchange resin (Corbin et al., 1975). All regulatory subunits, however, retain a common and well-defined domain structure that includes two tandem cAMP binding domains at the carboxy terminus, a site of dimer interaction at the amino terminus, and a proteolytically sensitive hinge region 90–100 residues from the amino terminus (Corbin et al., 1981; Takio et al., 1980, 1984; Taylor et al., 1981; Titani et al., 1984). The two tandem, nonidentical cAMP binding domains were originally distinguished kinetically on the basis of different dissociation rates for cAMP (Corbin et al., 1982) and by their capacity to discriminate between analogues of cAMP (Corbin et al., 1982; Døskeland et al., 1983). The more amino-terminal domain is referred to here as site A and the domain at the carboxy terminus at site B.

The tandem cAMP binding domains conserved in all regulatory subunits show sequence similarities with each other and with the catabolite gene activator protein (CAP) of *Escherichia coli* (Weber et al., 1982). On the basis of these sequence similarities, a reasonably detailed model of the structure for each cAMP binding domain in the regulatory subunits was predicted using the crystal structure of CAP as a template (Weber et al., 1987). Two features of this structure appear to be invariant: an Arg that is thought to interact electrostatically with the exocyclic oxygens of the cyclic phosphate ring of cAMP and a Glu which in CAP appears to hydrogen bond to the 2′OH of the ribose ring. These invariant Arg's in the type I regulatory subunit are Arg-209 and Arg-333 in site A and B, respectively. Replacement of this essential Arg in domain A of the R^I subunit with Lys is sufficient to abolish high-affinity cAMP binding to this site, and the cAMP binding properties of this regulatory subunit and of holoenzyme formed with this subunit have been described previously (Bubis et al., 1988). These previous studies were carried out in the absence of MgATP.

Unlike the type II holoenzymes, which contain an autophosphorylation site in the regulatory subunit (Rosen & Erlichman, 1975), the type I holoenzymes have a high-affinity binding site for MgATP (Hofmann et al., 1975). The hinge region of each regulatory subunit contains a sequence that resembles a substrate recognition site for the catalytic subunit (Takio et al., 1984; Taylor et al., 1990; Titani et al., 1984). The type II regulatory subunits contain an autophosphorylation site at this hinge region while the type I regulatory subunits contain a pseudophosphorylation site where the Ser is typically replaced with Ala or Gly. Binding of MgATP to the type I holoenzyme increases the K_a for activation by cAMP and the binding constant (K_d) for cAMP by 5–10-fold (Hofmann et

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‡ Present address: Department of Chemistry, Evergreen State College, Olympia, WA 98505.

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al., 1975). Since the type I holoenzyme is uniquely sensitive to MgATP, the properties of holoenzyme formed with the mutant regulatory subunit containing the R209/K replacement were investigated with specific emphasis on the role of MgATP. In particular, we have examined the effects of MgATP on (1) the cAMP binding properties of holoenzyme, (2) the activation of the mutant holoenzyme by cAMP, and (3) reassociation of the subunits to form holoenzyme.

Experimental Procedures

Materials. Chemicals were purchased from the following sources: cAMP, United States Biochemical; benzamidine, ATP, ADP, DNaseI, bovine serum albumin, and Luria broth (L-broth), Sigma; Hepes and ammonium sulfate, Fischer; [^{3}H]cAMP (30 Ci/mmol) and [^{3}H]ATP (30 Ci/mmol), New England Nuclear; glass filters (HA 0.45 μM), Millipore. Catalytic subunit was isolated from porcine heart according to Nelson and Taylor (1981). The construction of the vector replacing Arg-209 with Lys was reported previously (Bubis et al., 1988).

Protein Purification. E. coli strain 222 was transformed with pLST2(R209/K) and grown in L-broth containing ampicillin (50 μg/mL) for 24 h at 37 °C as described previously (Bubis et al., 1988; Saraswat et al., 1986). Cells from 4-L of culture were quickly chilled to 4 °C, collected by centrifugation and resuspended in 49 mL of lysis buffer. After the cells were ruptured by two passes through a French pressure cell, the particulate material was removed by centrifugation at 10000*g* for 20 min. The resulting supernatant was diluted to a conductivity below 1 mS and applied to a DE-23 column (23 cm × 30 cm) equilibrated with 10 mM potassium phosphate, 1 mM EDTA, and 5 mM β-mercaptoethanol, pH 6.5. After being washed with this buffer, the regulatory subunit was eluted with an 800-mL linear salt gradient (0–200 mM NaCl) in the same buffer. All protein purification steps were carried out at 4 °C.

Contaminating proteins or proteolytic fragments, when present, were removed by gel filtration on a Pharmacia G-150 column (1 × 50 cm) equilibrated in 10 mM potassium phosphate (pH 6.5), 1 mM EDTA, 5 mM β-mercaptoethanol, and 0.5 M NaCl. Protein concentrations were determined by the Bradford assay (Bradford, 1976) and confirmed by [^{3}H]cAMP binding (Døskeland & Øgreid, 1988). The purity of the proteins was monitored by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis followed by staining with Coomassie R-250. Gel electrophoresis was run according to Laemmli (1970) using 12.5% acrylamide.

Formation of Holoenzyme. Holoenzyme was formed by dialysis of the regulatory subunit with a 10% molar excess of purified catalytic subunit isolated from porcine heart as described previously (First & Taylor, 1988). After dialysis in buffer (20 mM potassium phosphate, pH 6.5, and 5 mM β-mercaptoethanol) containing 1 mM MgATP, excess catalytic subunit was removed with CM-Sepharose. Catalytic activity was assayed by using a coupled spectrophotometric method (Cook et al., 1982) in the presence and absence of 1 μM cAMP using the following peptide as substrate: L-R-R-A-S-L-G. This peptide substrate was synthesized by the UCSD Peptide and Oligonucleotide Facility. Formation of holoenzyme was confirmed by this assay as well as by visualizing the protein bands following SDS-polyacrylamide gel electrophoresis.

In order to determine the rate of holoenzyme formation, purified regulatory subunit saturated with cAMP was mixed with equimolar amounts of catalytic subunit in dialysis bags and added to 2 L of buffer in the presence and absence of 1 mM MgATP. At the indicated time points, aliquots were removed, and the extent of holoenzyme formation was determined by comparing catalytic activity in the presence and absence of 10^{-4} M cAMP. Holoenzyme with no bound MgATP was prepared in two different ways, either by dialyzing the regulatory subunit with a 10% molar excess of catalytic subunit, based on the R monomer, without using MgATP in the dialysis buffer for at least 4 days at 4 °C or by removing MgATP from the holoenzyme/MgATP complex by exhaustive dialysis for at least 48 h against buffer containing 2 mM EDTA. Both methods yielded identical proteins.

K_a for Dissociation of Holoenzyme. The K_a(cAMP) was determined at room temperature by measuring the fraction of maximal catalytic activity present in holoenzyme as a function of cAMP concentration. In a typical experiment, 350 μL of 25 nM holoenzyme was preincubated for 20–30 min at room temperature with various cAMP concentrations ranging from 1 nM to 500 μM. Aliquots (150 μL) were mixed with 850 μL of the spectrophotometric assay solution, and the decrease of NADH absorption was followed for 2 min. All assays were carried out in duplicate.

MgATP Binding. The affinity of the holoenzyme for MgATP was measured by ammonium sulfate precipitation after incubation at room temperature with varying concentrations of [^{3}H]ATP using the method described by Døskeland and Øgreid for cAMP binding (Døskeland & Øgreid, 1988). Holoenzyme (2–20 nM) was in 100 mM MOPS, pH 7.0, 100 mM KCl, 10 mM $MgCl_2$, and 0.5 mg/mL bovine serum albumin. Measurements at equilibrium were obtained after a 3-h incubation.

cAMP Binding and Exchange. cAMP affinity was measured in the same buffer used for MgATP binding. After incubation with labeled cAMP and varying concentrations of [^{3}H]cAMP, the extent of cAMP bound was determined by the filter binding assay following precipitation with ammonium sulfate (Døskeland & Øgreid, 1988). Off rates for bound cAMP were measured in buffer containing either low salt (0.15 M NaCl) or high salt (3.2 M NaCl). Regulatory subunit (1 nM) was saturated with [^{3}H]cAMP and then added to buffer containg 0.5 mg/mL bovine serum albumin, 5 mM β-mercaptoethanol, and 0.1 mM cold cAMP. At the designated time points, aliquots were removed, and labeled cAMP still bound to regulatory subunit was measured by the ammonium sulfate filtration assay.

Results

ATP Binding. As shown previously, the mutant regulatory subunit, R209/K, is still capable of reassociating with the catalytic subunit to form holoenzyme (Bubis et al., 1988). Previous studies also established that this holoenzyme in the absence of MgATP was dissociated in the presence of cAMP under conditions where only cAMP binding site B was occupied (Bubis et al., 1988). Since the native type I holoenzyme has a high-affinity binding site for MgATP, it was important to establish whether this high-affinity binding site also was retained in holoenzyme formed with the mutant regulatory subunit. Holoenzyme formed with the mutant regulatory subunit (R209/K) still bound MgATP with a high affinity similar to that seen for holoenzyme formed with the wild-type regulatory subunit. The observed K_d for MgATP of 15 nM was within experimental error of the K_d of 10 nM observed for the native holoenzyme. The Hill coefficient of 1.5 also was comparable to the native holoenzyme.

Effect of MgATP on cAMP Binding. Since one of the known consequences of MgATP binding to holoenzyme is a decrease in the affinity of holoenzyme for cAMP, the effect of MgATP on cAMP binding to the mutant holoenzyme was

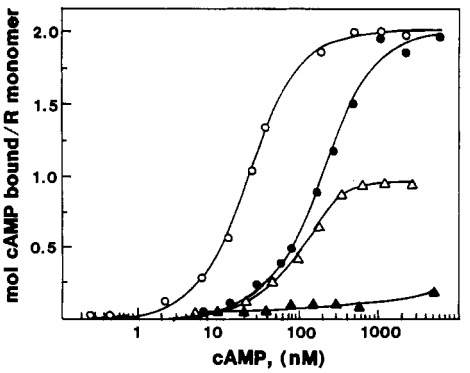

FIGURE 1: Effect of MgATP on cAMP binding. Circles (○, ●) correspond to wild-type holoenzyme, and triangles (△, ▲) refer to holoenzyme formed with the R209K mutant R subunit. Open and closed symbols correspond to measurements made in the absence and presence of MgATP.

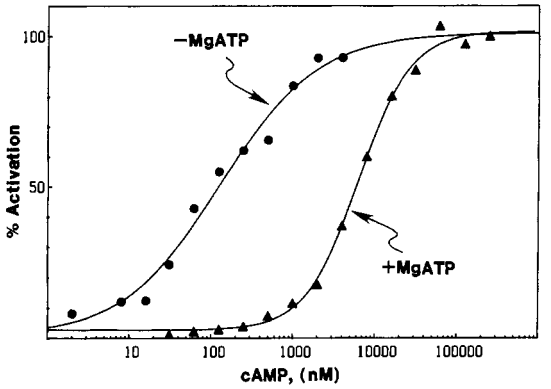

FIGURE 2: Effect of MgATP on activation of R209K mutant holoenzyme. Activity was measured in the absence (●) or presence (▲) of 10 μM MgATP.

characterized. In the case of wild-type holoenzyme, the addition of MgATP causes a 5–10-fold increase in the K_d for cAMP from 20 to 110 nM. When MgATP was added to holoenzyme formed with the mutant regulatory subunit (R209/K), the capacity of this complex to bind cAMP with a high affinity was shifted even further (Figure 1). The K_d(cAMP) preincubated in the absence of MgATP was 100 nM. It was not possible to measure cAMP binding directly in the presence of MgATP using this method at cAMP levels above 5 μM due to the large background at these higher levels of [^{3}H]cAMP.

Activation of the Holoenzyme. By lowering the affinity of the holoenzyme for cAMP, MgATP effectively increases the activation constant (K_a) for cAMP as well. Our earlier results established unambiguously that the mutant regulatory subunit (R209/K) only binds cAMP with a high affinity to site B, and binding to site B is sufficient to activate the holoenzyme in the absence of MgATP (Bubis et al., 1988). In the absence of MgATP, the K_a(cAMP) is somewhat higher for the mutant holoenzyme (130 nM) than the K_a for the native holoenzyme (30 nM). This value was determined by incubating holoenzyme in the absence of MgATP with varying concentrations of cAMP and then adding aliquots to the assay mixture (Figure 2). On the other hand, when holoenzyme is preincubated in the presence of MgATP as well as cAMP, the holoenzyme is very insensitive to activation by cAMP as was suggested by the binding studies described above. As seen in Figure 2, under these conditions, the K_a(cAMP) for activation of the holoenzyme is 6.5 μM versus 115 nM for the wild-type holoenzyme.

cAMP Exchange Rate. The two cAMP binding sites in the

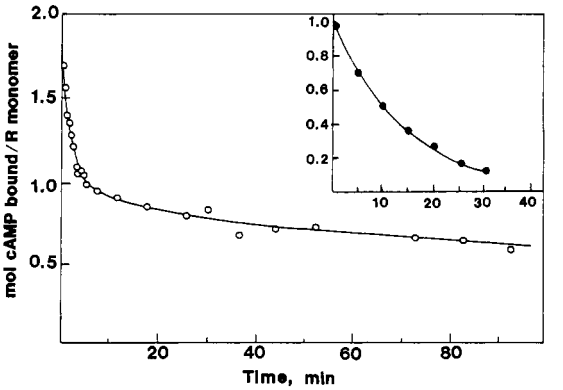

FIGURE 3: Rate of cAMP dissociation from wild-type and R209K mutant holoenzyme. The rate of dissociation of the wild-type enzyme (○) at 30 °C in the presence of high salt (3.2 M NaCl) is shown. In the inset (●) is the off rate of cAMP from the mutant holoenzyme.

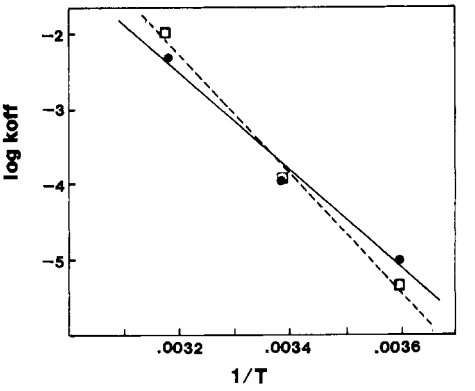

FIGURE 4: Arrhenius plot of the rate constants for the mutant (R209K) regulatory subunit. The squares (□) correspond to high-salt conditions (3.2 M NaCl); the circles (●) correspond to measurements made in the presence of low salt (0.15 M KCl).

native regulatory subunit can be distinguished readily by their corresponding exchange rates for cAMP (Corbin et al., 1982; Døskeland et al., 1983; Øgreid et al., 1983). This biphasic exchange rate is exaggerated by high-salt (3.2 M NaCl) conditions which cause the rapid exchange rate to increase and the slow exchange rate to decrease even further (Figure 3). The initial rapid exchange rate ($t_{1/2}$ = 1.5 min in high salt at 30 °C) represents the exchange of cAMP bound to site A, while the slower exchange rate ($t_{1/2}$ = 120 min) represents the exchange of cAMP bound to site B. The corresponding exchange rates under more physiological conditions (0.15 M NaCl) are 4 and 45 min, respectively.

In contrast to this biphasic pattern seen for the dissociation of cAMP from the wild-type regulatory subunit, the R209K mutant regulatory subunit shows a monophasic and relatively rapid cAMP exchange pattern under all salt and temperature conditions (Figure 3, inset). The temperature dependency of the exchange rate is shown in Figure 4. At 30 °C under high-salt conditions, the half-life for the exchange is 10.3 min, and the half-life for exchange under low-salt conditions is 18 min. At room temperature, 21–22 °C, the half-life for the exchange of [^{3}H]cAMP is the same under all salt conditions. At temperatures below 22 °C, the exchange rate is slower in high salt than in low salt. However, for the mutant R subunit, at high temperatures the exchange rate is actually faster in high salt than in low salt. This contrasts strikingly from the wild-type regulatory subunit where the exchange rate is consistently slower for site B in high salt at all temperatures (Døskeland & Øgreid, 1984).

Effect of the Catalytic Subunit and MgATP on the cAMP Exchange Rate. In the case of the native type I regulatory

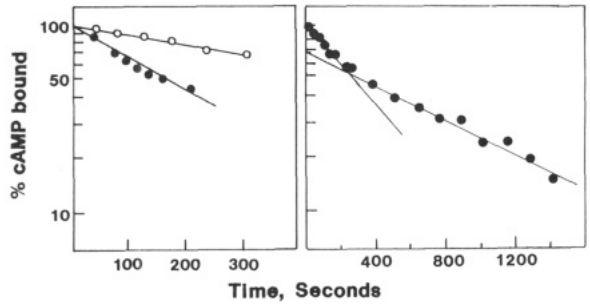

FIGURE 5: Dissociation of cAMP in the presence and absence of catalytic subunit. The left panel shows the dissociation of [^{3}H]cAMP in the presence (●) and absence (○) of catalytic subunit (10% molar excess of C subunit. Measurements were carried out at 30 °C in low salt (0.15 M KCl). The right panel shows the dissociation of cAMP when subsaturating concentrations of C subunit are used. In this case, the molar ratio of C to R monomer was 0.3 to 1.0. Measurements were carried out at room temperature, 21 °C, in low salt in the presence of 1 mM ATP and 10 mM $MgCl_2$.

subunit, the addition of free catalytic subunit in the absence of MgATP does not significantly alter the exchange rate for cAMP, unless a large molar excess (10-fold) of free catalytic subunit is added (Øgreid & Døskeland, 1983). On the other hand, catalytic subunit in the presence of MgATP causes a substantial increase in the exchange rate for bound cAMP. In the presence of MgATP and excess cAMP, the catalytic subunit facilitates this exchange of cAMP even when the catalytic subunit is present in substoichiometric amounts relative to the regulatory subunit (Chau et al., 1980). The half-maximal concentration of MgATP that is required to stimulate C-subunit-induced exchange of [^{3}H]cAMP from the native R is approximately 2.0 μM (Ringheim & Taylor, 1990).

In the case of the mutant regulatory subunit, catalytic subunit alone has a very substantial stimulatory effect on the exchange of cAMP (Figure 5). If MgATP is added, the cAMP exchange is increased further. For the mutant regulatory subunit, this effect also is not catalytic. If substoichiometric amounts of C subunit with ATP are added, the exchange reaction becomes biphasic, with an initial rapid exchange burst proportional to the amount of C subunit followed by a much slower exchange rate. The addition of 1 mM ATP/5 mM $MgCl_2$ without C subunit has no effect on the cAMP exchange rate of any R subunit (data not included).

Holoenzyme Formation Rate. The rate at which regulatory subunit saturated with cAMP and catalytic subunit recombines to form holoenzyme is greatly dependent on the presence or absence of MgATP in the case of wild-type regulatory subunit. As seen in Figure 6, catalytic subunit alone does not reassociate readily with the regulatory subunit in the absence of MgATP, while in the presence of MgATP reassociation is facilitated significantly. In contrast, catalytic subunit reassociates with the mutant regulatory subunit (R209/K) at a greatly accelerated rate relative to the wild-type regulatory subunit either in the presence or in the absence of MgATP. Under all conditions, the mutant regulatory subunit formed holoenzyme much faster than the wild-type protein. Holoenzyme formation was nearly complete as soon as measurements could be taken and did not appear to require dialysis to remove the released cAMP. In contrast, for the wild-type regulatory subunit, there is competition for the released cAMP to either rebind or dialyze.

The lack of dependence on MgATP for holoenzyme formation also is seen qualitatively in Figure 7. In this case, the regulatory subunit was dialyzed with a 10% molar excess of catalytic subunit at 4 °C for 24 h in the presence and absence of MgATP. At this point, any remaining free catalytic subunit

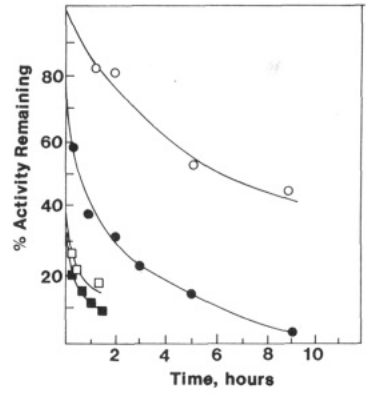

FIGURE 6: Formation of holoenzyme in the presence and absence of MgATP. The circles (○, ●) correspond to wild-type holoenzyme while the squares (□, ■) refer to the mutant holoenzyme. Open and closed symbols correspond to the formation of holoenzyme in the absence and presence of MgATP, respectively.

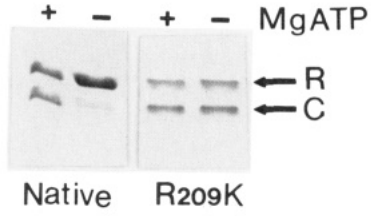

FIGURE 7: Formation of holoenzyme in the presence and absence of MgATP. Regulatory subunit and catalytic subunit (1:1.1 molar ratio) were dialyzed in the absence (−) and presence (+) of 10 μM MgATP. After 24 h, any residual free catalytic subunit was removed by cation-exchange chromatography, and the residual solution was electrophoresed in order to evaluate the extent of holoenzyme formation.

was bound to CM-Sephadex, while holoenzyme and free regulatory subunit remain in solution. The supernatant was subjected to SDS-polyacrylamide gel electrophoresis. While very little holoenzyme forms with the wild-type regulatory subunit under these conditions in the absence of MgATP, holoenzyme formation is complete with the mutant regulatory subunit whether or not MgATP is present.

Concentration of MgATP Needed To See the Shift in K_d(cAMP). Previous results established that the half-maximal concentration of MgATP required to shift the K_d(cAMP) for the wild-type holoenzyme was 2 μM, thus leading to the hypothesis that it was the C·MgATP complex that was required for reassociation with the cAMP-saturated R subunit (Ringheim & Taylor, 1990). As was seen in Figure 2, the K_a for the activation of the mutant holoenzyme by cAMP is also increased significantly in the presence of MgATP. In contrast to the wild-type holoenzyme, the concentration of MgATP required to cause this shift in K_a is much lower. As seen in Figure 8, at a concentration of 100 nM cAMP, the half-maximal concentration of MgATP required to shift the K_d(cAMP) is 250 nM as opposed to 3 μM for the wild-type holoenzyme. These results confirm that holoenzyme formation in this mutant holoenzyme that does not contain cAMP bound to site A does not require the C·MgATP complex since the K_m(MgATP) and K_d(MgATP) for the free catalytic subunit are 11 and 10 μM, respectively (Bhatnager et al., 1983; Zoller & Taylor, 1979).

Discussion

The two major classes of cAMP-dependent protein kinase existing in eukaryotic cells differ primarily in their responses to MgATP. The type II holoenzymes contain an autophosphorylation site at the hinge region of the regulatory subunit, and binding of MgATP results in an immediate transfer of the γ-phosphate from ATP to Ser-95 in the hinge

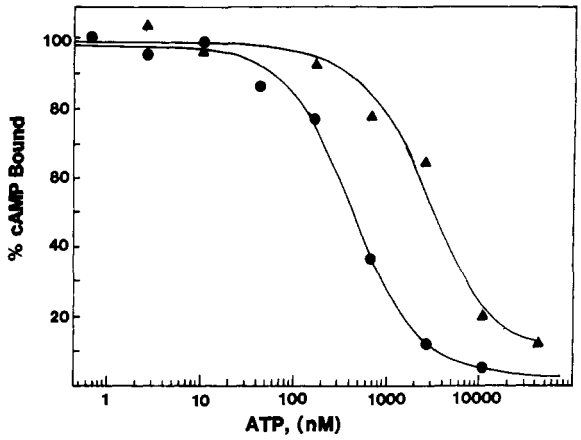

FIGURE 8: Concentration of MgATP required to shift the K_d for cAMP. Binding of cAMP was measured by the ammonium sulfate precipitation assay described under Experimental Procedures. The concentration of [^{3}H]cAMP used in this assay was 100 nM. Circles (●) represent holoenzyme formed with mutant regulatory subunit while triangles (▲) represent wild-type holoenzyme.

region of the regulatory subunit (Rosen & Erlichman, 1975; Titani et al., 1984). This occurs as an intramolecular event in the intact holoenzyme, indicating that the autophosphorylation site of the R^{II} subunit occupies the substrate binding site of the catalytic subunit in the holoenzyme and is poised to accept the γ-phosphate of ATP (Rangel-Aldao & Rosen, 1976). The type I holoenzymes do not contain an autophosphorylation site. The R^I subunit has a substrate recognition site located in the hinge region, but the potential site for autophosphorylation is replaced, typically, by an Ala (Takio et al., 1984; Taylor et al., 1990). Thus, the type I regulatory subunits have a pseudophosphorylation site. Replacement of this Ala with a Ser generates an autophosphorylation site, and, like the type II holoenzyme, phosphorylation of this mutant regulatory subunit occurs as an intramolecular event (Durgerian & Taylor, 1989). Instead of having an autophosphorylation site, the type I holoenzyme has a high-affinity binding site for MgATP (Hofmann et al., 1975). On the basis of kinetic mapping with analogues of ATP, it was concluded that the adenine portion of this binding site is most likely identical in the free catalytic subunit and in the holoenzyme while the environment surrounding the ribose and the phosphates differs in the free catalytic subunit and in the holoenzyme (Hoppe et al., 1977, 1978). In addition, since neither MgADP nor AMP binds to the holoenzyme with a high affinity (Hoppe et al., 1977), it is presumably the γ-phosphate that is critical. At the relatively high levels of MgATP found in cells, it is likely that under physiological conditions the type I holoenzyme exists almost exclusively as a complex with MgATP.

MgATP plays at least two functional roles in the native enzyme. First is its role in stabilizing the holoenzyme and making it less responsive to cAMP. Typically the K_a(cAMP) and K_d(cAMP) are increased approximately 5–10-fold in the presence of MgATP (Hofmann et al., 1975; Ringheim & Taylor, 1990). MgATP also plays a significant role in the reassociation of the catalytic subunit with the cAMP-saturated regulatory subunit. Rapid and stable dissociation of cAMP from the regulatory subunit requires both catalytic subunit and MgATP (Chau et al., 1980; Øgreid & Døskeland, 1983). Since the K_d(MgATP) that is required to produce a shift in K_c(cAMP) is 5 μM, we predicted previously that the C-(MgATP) binary complex is required to dissociated cAMP from the regulatory subunit (Ringheim & Taylor, 1990).

The results described here explore the role of MgATP in stabilizing the holoenzyme complex and in promoting the dissociation of bound cAMP in a mutant form of the regulatory subunit where cAMP binding to site A is impaired by a point mutation at the active site. As discussed earlier, two residues, an Arg and a Glu, are essential features for each cAMP binding site, based on the CAP model (Weber et al., 1987). Both are conserved in every cAMP binding site. Chemical evidence supports the conclusion that the Arg plays a particularly important role since analogues which contain sulfur substituants at the axial and equatorial positions of the cyclic phosphate ring function as agonists and antagonists, respectively, of cAMP (DeWit et al., 1984; Rothermel & Botelho, 1988). This chemical evidence is reinforced by replacement of Arg-209 in site A of the R^I subunit with Lys, a mutation that abolishes high-affinity binding of cAMP to site A. In the absence of MgATP, this mutant regulatory subunit forms holoenzyme, and in the presence of cAMP, this holoenzyme dissociates into an R dimer and two free and active catalytic subunits with a K_d(cAMP) that is somewhat greater than the native protein (Bubis et al., 1988). However, in the presence of MgATP, this protein behaves quite differently.

Consider first the role of MgATP in stabilizing the holoenzyme and lowering its sensitivity to cAMP. Holoenzyme formed with the mutant regulatory subunit still binds MgATP with a high affinity in a manner that is very similar to the native holoenzyme. The K_d(MgATP) values for the wild-type and mutant holoenzymes are 10 and 15 nM, respectively. For the native holoenzyme, MgATP causes a 5–10-fold shift in the K_d(cAMP). The capacity of MgATP to stabilize the holoenzyme is enhanced considerably when Arg-209 in domain A of the regulatory subunit is replaced with Lys. In this case, MgATP locks the entire complex into a form that cannot be activated readily by cAMP. Neither site A nor site B can bind cAMP at submicromolar concentrations when MgATP is bound to the holoenzyme. It is difficult to measure the binding constant for cAMP directly at these high concentrations of cAMP, but the K_d(cAMP) for the mutant holoenzyme is well above 1 μM. The K_a(cAMP) is increased 60-fold. Thus, under physiological conditions in the cell, we predict this mutant holoenzyme would be maintained in an inactive holoenzyme form since intracellular cAMP levels do not typically exceed 1 μM (Robinson et al., 1971).

The regulatory subunit can typically exist in two different conformational states—the cAMP-bound state (B form) and the holoenzyme state (H form):

$$\underset{\text{H form}}{R_2C_2(MgATP)_2} + 4cAMP \quad R_2(cAMP)_4 + 2C(MgATP)_{\text{B form}}$$

The native regulatory subunit requires that both cAMP binding sites be occupied in order to dissociate the holoenzyme complex and stabilize the cAMP-bound conformation. Dissociation proceeds through a quaternary intermediate complex containing R, C, MgATP, and cAMP (Builder et al., 1980; Chau et al., 1980). On the basis of kinetic evidence, it is predicted that site B, but not site A, is exposed in the holoenzyme and accessible to cAMP (Øgreid & Døskeland, 1981). According to this model, summarized diagrammatically in Figure 9, binding of cAMP to site B causes a conformational change that makes site A more accessible. Binding of cAMP to site A then becomes the key step that promotes dissociation of the holoenzyme complex. The mutant R subunit in the absence of MgATP shows more flexibility. In this case, occupancy of site B alone is sufficient to dissociate the complex and presumable convert the regulatory subunit into a conformation that more closely resembles the B form. The unoccupied A domain apparently is no longer sufficient in the

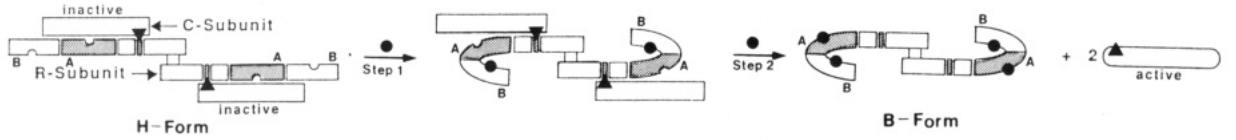

FIGURE 9: Activation of the type I holoenzyme. The H form shows the holoenzyme form where the triangle (▼) indicates bound MgATP with the γ-PO_4 coming close to the hinge region of the regulatory subunit. Both the hinge and site A are shaded.

absence of MgATP to form a tight complex with the catalytic subunit. In contrast, in the presence of MgATP, site A is apparently locked into its holoenzyme conformation very tightly, and, in addition, site B is now shielded and can no longer bind cAMP readily. Activation in the presence of MgATP exhibits a Hill coefficient of 1.6, suggesting that activation under these conditions may actually require occupancy of both cAMP binding sites.

It was predicted previously, based on sequence identity with CAP, that Arg-209 in cAMP binding site A interacts with the exocyclic oxygens in the cyclophosphate ring of cAMP when the cyclic nucleotide is bound (Bubis et al., 1988; Weber et al., 1987). Subsequent binding studies with analogues of cAMP led us to suggest, furthermore, that the guanidinium side chain of Arg-209 very likely forms two stable bonds with cAMP, in contrast to the single ionic interaction observed in the crystal structure of CAP (Dostmann et al., 1990). If this prediction is valid, then replacement of Arg-209 with Lys would eliminate 50% of the ionic pairing and is consistent with the significant observed reduction in affinity for cAMP. Unfortunately, we cannot predict what role Arg-209 plays in the holoenzyme complex when cAMP is not bound. Our results suggest that this highly conserved residue not only is important for binding cAMP but also plays a major role in the holoenzyme and is particularly important for communicating the conformational changes that result from cAMP binding.

Since replacement of Arg-209 with Lys abolishes high-affinity cAMP binding, it is clear that simply retaining a positive charge at this position alone is not sufficient. The steric and spatial properties of the guanidinium group are essential, perhaps because it allows for two hydrogen bonds to chelate to two oxygens in the cAMP moiety. Replacement of the Arg-209 with Lys, however, appears to preferentially stabilize the holoenzyme when MgATP is bound. Since no crystal structure of CAP in the absence of cAMP is available, we do not have a model to explain the role of Arg-209 in the cAMP-free form of the regulatory subunit. Whatever that role might be, it is strengthened by the presence of MgATP and cannot be easily disrupted when cAMP binding to site A is impaired. Without a model for the cAMP-free structure, we cannot easily predict the conformational changes that ensue as a direct consequence of cAMP binding. However, on the basis of these results, we propose that the changes could be accounted for by a series of salt bridges. We know that the essential sites for interaction between the regulatory and catalytic subunits are associated with the A domain and the hinge region since a mutant with the B domain deleted entirely is fully functional (Saraswat et al., 1988). Both cAMP binding and holoenzyme activation of holoenzyme formed with this deletion mutant are, furthermore, inhibited in the presence of MgATP in a manner very analogous to the wild type holoenzyme.

MgATP also is required, together with the catalytic subunit, for the rapid reassociation of the native regulatory and catalytic subunits. The mutant regulatory subunit, however, does not show this requirement for MgATP. It reassociates readily with the catalytic subunit even in the absence of MgATP. MgATP affords only a marginal increase in the rate of holoenzyme formation. The off rates for cAMP also reflect this lack of dependency on MgATP. The off rates for the mutant regulatory subunit are rapid, monophasic, and less sensitive to ionic strength than the wild-type holoenzyme. More importantly, and in contrast to the wild-type regulatory subunit, the off rate is not enhanced significantly in the presence of MgATP. From these results, we conclude that the role of C·MgATP in the reassociation process is to release cAMP from site A and prevent its rebinding. In this mutant R subunit with an impaired ability to bind cAMP at site A, this requirement for MgATP is abolished since site A is unoccupied. The next step will be to determine the consequences of introducing a parallel mutation into site B. Does replacement of Arg-333 with Lys have a similar effect on cAMP binding, and what difference, if any, does MgATP have on cAMP binding and holoenzyme activation?

Registry No. MgATP, 1476-84-2; cAMP, 60-92-4; Arg, 74-79-3; protein kinase, 9026-43-1.

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Pulsed EPR Analysis of Tartrate Dehydrogenase Active-Site Complexes†

Peter A. Tipton* and Jack Peisach
Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461
Received July 10, 1990; Revised Manuscript Received October 8, 1990

ABSTRACT: Mn^{2+}·tartrate dehydrogenase·substrate complexes have been examined by electron spin echo envelope modulation spectroscopy. The occurrence of dipolar interactions between Mn^{2+} and ^2H on [^2H]pyruvate and [4-^2H]NAD(H) confirms that Mn^{2+} binds at the enzyme active site. The ^2H signal arising from labeled pyruvate was lost if the sample was incubated at room temperature, indicating that the enzyme catalyzes exchange between the pyruvate methyl protons and solvent protons. Mn–^{133}Cs dipolar coupling was also observed, which suggests that the monovalent cation cofactor also binds in the active site. The tartrate analogue oxalate was observed to have a significant effect on the binding of NAD(H). Oxalate appears to constrain the binding of NAD(H) so that the nicotinamide portion of the cofactor is held in close proximity to Mn^{2+}. Spectra of enzyme complexes prepared with (*R*)-[4-^2H]NADH showed a more intense ^2H signal than analogous complexes prepared with (*S*)-[4-^2H]NADH, demonstrating that the *pro-R* position of NADH is closer to Mn^{2+} than the *pro-S* position and suggesting that tartrate dehydrogenase is an A-side-specific dehydrogenase. Oxalate also affected Cs^+ binding; the intensity of the ^{133}Cs signal increased in the presence of oxalate, which suggests that oxalate facilitates binding of Cs^+ to the active site or that Cs^+ binds closer to Mn^{2+} when oxalate is present. In addition to signals from substrates, electron spin echo envelope modulation spectra revealed ^{14}N signals that arose from coordination to Mn^{2+} by nitrogen-containing ligands from the protein; however, the identity of this ligand or ligands remains obscure.

Tartrate dehydrogenase is induced in *Pseudomonas putida* by growth on (+)-tartrate as the sole carbon source and catalyzes the first step in the catabolism of tartrate (Kohn & Jakoby, 1968), the NAD^+-dependent oxidation of (+)-tartrate to oxaloglycolate. TDH[1] has also been found to catalyze two other reactions, the oxidative decarboxylation of D-malate and the net nonoxidative decarboxylation of *meso*-tartrate (Tipton & Peisach, 1990). All these reactions also require Mn^{2+} and K^+ as cofactors. There are a variety of mechanisms through which Mn^{2+} potentially could participate in these reactions, for example, by lowering the p*K* of the hydroxyl group at the carbon center being oxidized, by facilitating decarboxylation

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* Address correspondence to this author at his present address: Department of Biochemistry, M121 Medical Sciences Building, University of Missouri—Columbia, Columbia, MO 65212.

[1] Abbreviations: TDH, tartrate dehydrogenase; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; ESE, electron spin echo; TEA, triethanolamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

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