Effect of cAMP and ATP on the Reassociation of Phosphorylated and Nonphosphorylated Subunits of the cAMP-dependent Protein Kinase from Bovine Cardiac Muscle*

(Rafael Rangel-Aldao and Ora Mendelsohn Rosen)

From the Departments of Molecular Pharmacology and Medicine, Albert Einstein College of Medicine, Bronx, New York 10461

Self-phosphorylation of the bovine cardiac muscle adenosine 3',5'-monophosphate (cAMP)-dependent protein kinase results in retardation of the rate of reassociation of its isolated subunits in the absence of cAMP (Rangel-Aldao, R., and Rosen, O. M. (1976) J. Biol. Chem. 251, 3375-3380). We have now studied the extent of reassociation of phosphorylated and nonphosphorylated cAMP-binding protein (R2) with the catalytic subunits (C) in the presence of cAMP and ATP using chromatography on ω-aminohexyl agarose to resolve the various forms of the enzyme, and a purified cyclic nucleotide phosphodiesterase to bring about changes in the concentration of cAMP. The following observations were made. (a) The extent to which R2 (phosphorylated and nonphosphorylated) and C reassociate is much smaller in the presence than in the absence of cAMP. (b) Both forms of R2 reassociate completely with catalytic subunits when the added cAMP had been hydrolyzed by cyclic nucleotide phosphodiesterase. (c) The unphosphorylated cAMP-binding protein achieved 50% reassociation at a concentration of cAMP 10-fold higher than its phosphorylated counterpart. (d) Reassociation of both the phosphorylated and unphosphorylated protein kinase involved the formation of a new species of enzyme prior to the formation of the holoenzyme tetramer (R2C3). This putative intermediate was assigned an RKC structure; it possessed both regulatory and catalytic subunits, a ratio of R to C activities twice that observed in the tetramer (R2C3), and a molecular weight consistent with this subunit composition (137,000). (e) Millimolar concentrations of ATP limited the extent of reassociation of both phosphorylated and unphosphorylated R2 with C. This effect was not dependent upon divalent cations and did not involve a phosphotransferase reaction. Similar inhibitions were observed with ADP, AMP, and adenosine. At the same concentration, these latter compounds also inhibited the phosphotransferase activity of protein kinase, suggesting that interaction with the catalytic subunit of protein kinase may be involved in their ability to inhibit reassociation.

It is concluded that in the presence of physiological concentrations of ATP and cAMP, the extent to which protein kinase is reassociated to the inactive holoenzyme may be significantly affected by the state of phosphorylation of R2.

The principal soluble adenosine 3',5'-monophosphate-dependent protein kinase of bovine cardiac muscle is a tetramer consisting of one cAMP-binding protein dimer (R2) and two monomeric catalytic subunits (C) (1). Cyclic AMP activates the enzyme by dissociating it into R and C (2-6). The catalytic subunits residing in the holoenzyme catalyze the transfer of 3P from γ-32P[ATP to 2 specific seryl residues in R (7, 8). This phosphate can be removed after dissociation of the holoenzyme by cAMP, either by the action of phosphoprotein phosphatases (9) or by reversal of the phosphotransferase reaction in the presence of ADP (10). Both forms of protein kinase, phosphorylated and nonphosphorylated, have been shown to bind cAMP equally well and to be completely dissociated by cAMP (11, 12). Both can reassociate completely upon removal of cAMP (7, 11, 12). However, in the absence of cAMP, the phosphorylated R reassociates with C at least 5-fold slower than the nonphosphorylated R (10). The objective of this study was to extend these findings by analyzing the extent of reassociation of both forms of protein kinase in the presence of cAMP and ATP. The experiments were designed to test whether the state of phosphorylation of R affected the proportions of dissociated (active) and reassociated (inactive) kinase under conditions of changing cyclic nucleotide concentrations and in the presence and absence of millimolar ATP.

MATERIALS AND METHODS

γ-32P[ATP (>10 Ci/mmol) was purchased from Amersham/Searle; [3H]cAMP (50 Ci/mmol) and Omnifluor were obtained from New England Nuclear; other nucleotides and nucleosides were from Sigma Chemical Co.; protamine sulfate was from Eli Lilly, ω-aminohexyl agarose, from Miles-Yeda Laboratories. All other reagents were of the highest degree of purity commercially available. A culture of Serratia marcescens kilensis (ATCC 9986) was obtained...
from American Type Culture Collection. Polyethyleneimine cellulose-F plates (20 × 20 cm) were from E. Merck, Darmstadt, Germany.

Protein Kinase Assay—Protein kinase was assayed by a modification of the procedure of Rubin et al. (13). The reaction mixture (200 μL) contained 20 mM potassium phosphate buffer, pH 7.1, 10 mM MgSO₄, 50 μM [γ-32P]ATP (30 to 50 cpm/pmol), 10 mM dithiothreitol, 0.25 mg of protamine sulfate, 0.5 mg of bovine serum albumin and, when indicated, 20 μM cyclic AMP. Incubations were for 2 to 5 min at 20°. One unit of enzyme activity is defined as that amount catalyzing the transfer of 1 nmol of βγP from [γ-32P]ATP to protamine per min.

Cyclic AMP-binding Assay—Cyclic [3H]AMP binding was assayed according to Gilman (14) except that 50 mM potassium phosphate, pH 7.1, was substituted for 0.05 mM sodium acetate buffer, pH 4.0. Protein was measured by the method of Lowry et al. (15) using bovine serum albumin as standard.

Preparation of Protein Kinase—Protein kinase was isolated from bovine cardiac muscle (13). The purified enzyme had a specific activity of 800 nmoles of ATP transformed to protamine/min/mg of protein at 37° and bound 2 mol of cyclic [3H]AMP/mol of holoenzyme. Preparations of purified protein kinase, designated here as nonphosphorylated, vary in their ability to accept phosphate (1.0 to 1.9 mol/mol of holoenzyme) (7, 11). The particular preparation used in these studies (incorporated 1.1 mol of [32P]ATP from [γ-32P]ATP per mol of enzyme). It was estimated to be at least 90% pure by polyacrylamide gel electrophoresis in the absence (16) or presence (17) of sodium dodecyl sulfate and did not contain detectable cyclic nucleotide phosphodiesterase activity. Calculations of the millimolar concentrations of cyclic AMP bound in experiments are based upon molecular weights of 174,000 for the holoenzyme, 98,000 for the cyclic AMP-binding protein dimer and 38,000 for the catalytic subunit (18).

Phosphorylation of Protein Kinase—Self-phosphorylation was carried out as described previously (11). The reaction mixture (100 μL) was incubated at 4° for 1 h and contained 100 μg of purified protein kinase, 50 mM potassium phosphate buffer, pH 7.1, 10 mM MgSO₄, and 0.2 mM [γ-32P]ATP (1000 to 2000 cpm/pmol). Purified, unphosphorylated enzyme (7) was treated in the same way as the enzyme undergoing self-phosphorylation except that ATP was omitted from the reaction mixture. After phosphorylation was complete, both enzymes were dialyzed against four changes (1 liter each) of 50 mM potassium phosphate buffer, pH 7.1, containing 4 mM 2-mercaptoethanol for a total of 16 h.

Isolation of Protein Kinase Subunits—Dissociation of the holoenzyme and isolation of subunits free of cyclic nucleotides were carried out as reported (11) using [3H]cAMP to dissociate protein kinase and ω-aminohexyl agarose (19) to resolve the subunits.

Assay for Cyclic Nucleotide Phosphodiesterase—Cyclic nucleotide phosphodiesterase activity was assayed using thin layer chromatography of polyethyleneimine cellulose plates (11). The reaction mixture (25 μL) contained 100 mM Tris/HCl buffer, pH 8.0, and 1 mM cyclic [3H]AMP. The CAMP remaining after incubation with cyclic nucleotide phosphodiesterase and protein kinase (See Figs. 1 and 5) was determined using the same chromatographic method.

Reassociation of Protein Kinase Subunits—Cyclic nucleotide phosphodiesterase was purified 100-fold from Serrotaia marcescens kiliensis according to the procedure of Okabayashi and Ide (20). Its specific activity, under the conditions of our experiments, was 2.5 μmol of [3H]AMP hydrolyzed/min/mg of protein. The enzyme preparation was devoid of protein kinase and 5'-nucleotidase activities. As reported by Okabayashi and Ide (20), it was fully active in the absence of divalent cations and was inhibited only to 5% by the concentration (1 mM) of adenine nucleotides used in our studies.

Polyacrylamide Gel Electrophoresis—Electrophoresis in 5% polyacrylamide was performed according to Davis (16). Samples were applied in 100 μL of 35% glycerol, pH 7.0, and electrophoresis was performed at 1 mA/gel for 5.5 h at 4°. Following electrophoresis, gels were stained in a solution containing 0.2% Coomassie brilliant blue, 45% methanol, and 10% glacial acetic acid. Destaining was carried out in 25% methanol containing 10% glacial acetic acid.

Sedimentation in Sucrose Density Gradients—Sedimentation in sucrose density gradients were performed according to Martin and Ames (21). Samples (100 μL) were applied to 4.8-m1 linear gradients of 9 to 20% sucrose in 50 mM potassium phosphate buffer, pH 7.1, containing 4 mM 2-mercaptoethanol and 1 mg/ml of bovine serum albumin. Centrifugation was performed at 40,000 rpm at 4° for 16 h.

The reaction mixture was then applied to a column (5 x 20 mm) containing 200 μL of ω-aminohexyl agarose equilibrated at 23° with the buffer indicated above. The column was washed first with 1.5 ml of the same buffer to elute the catalytic subunit and subsequently with 1.5 ml of this buffer containing 0.2 M NaCl to elute the reassociated enzyme. Fractions (0.5 mL each) were collected into bovine serum albumin (final concentration 1 mg/ml) at 4° and assayed for protein kinase activity at 30° for 2 min in the presence and absence of 10 μM cAMP. The total activity of protein kinase applied to each column was 8 units. The recovery for each column varied from 86 to 93%. Reassociation was computed as previously detailed (11) by dividing the units of protein kinase activity eluted by 0.2 M NaCl by the total units of protein kinase recovered. The CAMP content of a 3-μl aliquot of the reaction mixture taken 10 s before application of the rest of the mixture to the minicolumn was estimated by thin layer chromatography (see "Materials and Methods"). The symbols are: O, phosphorylated protein kinase; ●, unphosphorylated protein kinase; △, reassociated protein kinase. The dashed line corresponds to reassociation obtained at time zero (see text).

RESULTS

Reassociation of Protein Kinase Subunits in the presence of cAMP—We have shown previously (11) that one can assay reassociation of the isolated subunits of protein kinase in the absence of cAMP using minicolumns of ω-aminohexyl agarose which rapidly resolve the catalytic subunit from the holoenzyme and R₂. This technique can also be used to follow the reassociation of subunits in the presence of cAMP if, after dissociation of the holoenzyme with the cyclic nucleotide, an excess of cyclic nucleotide phosphodiesterase is added to decrease the concentration of free cAMP. This kind of analysis allows a comparative study of the extent of reassociation of phosphorylated and unphosphorylated R₂ with C in the presence of varying concentrations of cAMP. As shown in Fig. 1, reassociation occurs concomitantly with a decrease in cAMP concentration; it does not occur in the absence of added cyclic nucleotide phosphodiesterase. It can be seen that as the cAMP concentration decreases, the extent to which R₂ has reassociated exceeds that of its phosphorylated counterpart. After 10 min of incubation with cyclic nucleotide phosphodiesterase.
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esterase, for example, the cAMP concentration is 20 \( \mu M \). Significant reassociation (65\%) of the nonphosphorylated \( R_e \) has occurred, whereas the phosphorylated cAMP-binding protein is only 25\% reassociated. This difference is magnified if one subtracts the 8\% apparent reassociation measured at zero time in the presence of cAMP. The phosphorylated enzyme is then reassociated only 17\% compared to 57\% for the nonphosphorylated form. The amount of protein kinase eluted as holoenzyme at time zero (8\%) was the same for both forms of protein kinase. This activity probably reflects undissociated holoenzyme rather than reassociated enzyme since it was not decreased by equilibrating the minicolumns with 0.4 to 1.0 mm cAMP. Concentrations of cAMP greater than 1 mm may be required to produce 100\% dissociation of the relatively high concentration of protein kinase (2.3 mm) used in these experiments. Alternatively, some molecules of protein kinase may have been damaged such that they can no longer be dissociated by cAMP. Fig. 1 also shows that phosphorylated \( R_e \) achieved 50\% reassociation at a cAMP concentration 10-fold lower (4 pm) than that required by \( R_p \). It should be noted in considering this data that the concentration of protein kinase used in this assay (2.3 mm) is higher than that calculated to be present in a muscle cell (0.2 to 1.0 mm) assuming a uniform distribution of the enzyme (22). We have determined that the \( K_m \) for cAMP of either phosphorylated or nonphosphorylated protein kinase at the concentration of enzyme used in this study is about 25 pm. The difference in the amounts of reassociated phosphorylated and nonphosphorylated \( R_e \) in the presence of cAMP was confirmed by electrophoretic analysis of the products of reassociation obtained after removal of unreacted catalytic subunit by the \( \omega \)-aminohexyl agarose minicolumns. In Fig. 2, we depict an experiment comparable to the one shown in Fig. 1 except that a higher concentration of protein kinase (6 mm) was used to ensure detection of the holoenzyme and \( R_e \) in the gels. At each concentration of cAMP, the reaction of C with \( R_e \) yielded more holoenzyme (\( R_eC \)) and less free \( R_e \) than the reaction of C with phosphorylated \( R_p \). Electrophoresis also revealed the presence of another protein migrating between the holoenzyme (\( R_eC \)) and \( R_e \). The amount of this protein was related to the extent of reassociation. It was present in low concentrations when the enzyme was almost completely dissociated, increased as reassociation approached 50\% and finally decreased as complete reassociation was attained. It followed the same sequence during reassociation of both forms of protein kinase suggesting that it was an intermediate in the reassociation process. The protein is not the catalytic subunit of protein kinase because free C was removed from the mixture before electrophoresis and C does not migrate to this position during electrophoresis (13). Since the catalytic activity eluted from the minicolumn prior to electrophoresis was stimulated by cAMP and the resin only binds C which is, in turn, bound to R (11), it was likely that the protein corresponded to some combination of \( R_e \) and C. To establish its identity, we performed an experiment (Fig. 3) similar to the one depicted in Fig. 2 using \( ^{32}P \)-labeled \( R_e \). A parallel gel was sliced and assayed for both catalytic activity and protein (\( R_e \)-bound) \( ^{32}P \). The activities of both the holoenzyme and the putative intermediate were stimulated by cAMP and therefore, had to contain the cyclic nucleotide-binding protein. However, the ratio of cyclic nucleotide binding to catalytic activity in the intermediate expressed as the ratio of \( ^{32}P \)-bound/unit of catalytic activity (assayed in the presence of cAMP) was twice that of \( R_eC_p \). This is compatible with an \( R_eC \) subunit composition. A molecule containing one catalytic subunit and the cyclic nucleotide-binding protein dimer should have a molecular weight of about 136,000 (18). To test this prediction, we took a mixture of \( R_e \) and C which contained a high molar ratio of \( R_e \) to C and, in the absence of cyclic nucleotides, subjected it to sedimentation in a sucrose gradient. The mixture containing a high proportion of \( R_e \) relative to C was obtained by dissociating the holoenzyme 85\% with cGMP and then isolating \( R_e \) containing a small amount of C by chromatography on \( \omega \)-aminohexyl agarose as described for the preparation of subunits (11). A parallel incubation prepared in the absence of bovine serum albumin showed an electrophoretic pattern similar to the zero time point for the nonphosphorylated protein kinase depicted in Fig. 2. Fig. 4 shows the behavior of this preparation of \( R_e \) and C in a sucrose density gradient. Only one symmetrical peak of catalytic activity was found with a sedimentation coefficient (\( s_{20,w} \)) of 5.8. The molecular weight, calculated on the assumption that the frictional ratio of the intermediate would be the same as that established for \( R_p \) and \( R_eC \) (1.6 (18)), was 137,000, consistent with that predicted for a molecule containing one cyclic nucleotide binding protein dimer (\( M_p = 98,000 \)) and one catalytic subunit (\( M_p = 38,000 \)).

Effect of ATP on Extent of Reassociation—Since protein kinase is exposed in vivo to millimolar concentrations of ATP (23-25), we studied the behavior of the dissociated protein kinase in the presence of 1 mm ATP, and monitored the extent of reassociation under conditions in which the free cAMP concentration was varied between 0.4 mm and 0.1 pm. As shown in Fig. 5, ATP delayed the onset of formation of reassociated protein kinase (compare Figs. 5 and 1). Both phosphorylated and nonphosphorylated forms were affected. In the first 10 min of the experiment depicted in Fig. 5, cAMP concentrations fell from 0.4 to 0.028 mm and only the nonphosphorylated \( R_e \) reassociated. Later on, between 10 and 15 min, when the cAMP concentration had fallen to 2 pm, the nonphosphorylated \( R_e \) was 85\% reassociated and the phosphorylated form was beginning to reassociate. The concentration of cAMP had to fall to less than 0.1 pm in order to achieve 50\% reassociation of phosphorylated \( R_e \) with C. In the absence of ATP, reassociation occurred at higher concentrations of cAMP than in the presence of ATP. The effect of ATP was not mediated by a protein kinase reaction since it occurred in the absence of Mg\(^{2+} \) and in the presence of 1 mm EDTA.\(^2\) Additionally, reassociation of \( ^{32}P \)-labeled \( R_e \) was also inhibited by ATP under conditions in which the phosphate groups on the subunit remained intact. The phosphorylase preparation used in this study contained no detectable ATPase or phosphatase activities and was not significantly inhibited by the nucleotides added to the incubations. Thus, ATP itself appeared to affect the extent of reassociation. Other adenine nucleotides and adenosine were also capable of inhibiting reassociation in the absence of Mg\(^{2+} \) (see Table I). Of the non-adene nucleotides tested, only GTP significantly inhibited reassociation. When the concentration of the nucleotides or adenosine was decreased to 0.1 mm, there was only slight (10\%) inhibition of reassociation. The inhibition of reassociation could be due to interaction with \( R_e \), C, or both. When the binding of cyclic \( ^{32}P \)AMP to both phosphorylated and nonphosphorylated protein kinases was assessed at the concentrations of cAMP that pertained during the reassociation experiments, the addition of 1 mm ATP was found to have no effect on the equilibrium

\(^2\) EDTA (1 mm) did not affect reassociation.
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binding of cAMP to either form of the enzyme. On the other hand, the compounds that affected the net reassociation of R with C (Table I) exerted inhibitory effects on the catalytic activity of both the holoenzyme (assayed in the presence (Table II) or absence of cAMP) and the isolated catalytic subunit (assayed in the absence of cAMP (Table II)). Similar results have been reported by Kuo et al. (26) and Miyamoto et al. (27). ADP and adenosine, the more potent of the inhibitors of protein kinase activity, were competitive antagonists of ATP exhibiting K values of 10 and 50 μM, respectively. Thus, interactions with an ATP site on C may be involved in their ability to inhibit reassociation of C with either R or phosphorylated R.

**DISCUSSION**

Self-phosphorylation of protein kinase has previously been shown to result in a decrease in the rate of reassociation of the isolated subunits of protein kinase in the absence of

![Fig. 2](image2.png)

**Fig. 2.** Analysis of reassociated protein kinase by polyacrylamide gel electrophoresis. Phosphorylated or nonphosphorylated protein kinase (30 μg) was treated with 0.4 mM [3H]-cAMP and cyclic nucleotide phosphodiesterase as indicated in the legend to Fig. 1 except that albumin was omitted from the reaction mixture and binding of cAMP to either form of the enzyme. On the other hand, the compounds that affected the net reassociation of R with C (Table I) exerted inhibitory effects on the catalytic activity of both the holoenzyme (assayed in the presence (Table II) or absence of cAMP) and the isolated catalytic subunit (assayed in the absence of cAMP (Table II)). Similar results have been reported by Kuo et al. (26) and Miyamoto et al. (27). ADP and adenosine, the more potent of the inhibitors of protein kinase activity, were competitive antagonists of ATP exhibiting K values of 10 and 50 μM, respectively. Thus, interactions with an ATP site on C may be involved in their ability to inhibit reassociation of C with either R or phosphorylated R.

**FIG. 3.** Subunit composition of the intermediate present during the process of reassociation of protein kinase. Protein kinase (25 μg) was incubated in a final volume of 12 μl for 5 min at 23° in 50 mM potassium phosphate buffer containing 10 mM MgSO4 and 0.1 mM [γ-32P]ATP (2500 cpm/pmol). The mixture was then incubated with 0.4 mM cAMP at 23° for 5 min in a final volume of 25 μl. Cyclic nucleotide phosphodiesterase (1.6 μg in 2 μl of the above buffer) was then added and incubated for 10 min. The reaction was terminated and processed as indicated in the legend to Fig. 2. After completion of electrophoresis, the gel was cut into 1.0 mm slices and incubated with continuous shaking for 14 h at 4° in 200 μl of 50 mM potassium phosphate buffer, pH 7.1, containing 4 mM β-mercaptoethanol and 1 mg/ml of bovine serum albumin. Protein kinase activity in 50 μl of the eluate was assayed in the presence (●—●) of 10 μM cAMP. An aliquot (70 μl) of the eluate from each slice was then assayed for 32P in a scintillation mixture containing Triton X-100:toluene (30:70) and Omnifluor (4 g/liter) (○—○). The recovery of protein kinase activity and 32P from the gels was 75 and 60%, respectively. The unlabeled arrow indicates the position of the putative intermediate, R2C.

from the fractions containing the 0.2 M NaCl eluate. An aliquot of this eluate (25 μl) was immediately taken and admixed with bovine serum albumin to a final concentration of 1 mg/ml. The remainder was concentrated to 60 to 80 μl by dialysis against 5 liters of 60% glycerol for 60 min at 4°. The recovery of catalytic activity was 75 to 85%. The concentrated fractions from each column were then subjected to electrophoresis as described under "Materials and Methods." The gels were stained and scanned at 550 nm using a Gilford spectrophotometer with scanning and recording devices. Reassociation was quantitated as described in the legend to Fig. 1. In the scans, reading from right to left or top to bottom of the gel, the first peak is the cAMP-binding protein dimer, R2, the second peak is the putative intermediate, R2C, and the third peak is the holoenzyme, R2C2. The minor peak following R2C2 is a contaminant in the protein kinase preparation. A, reassociation of nonphosphorylated R2 and C; B, reassociation of phosphorylated R2 and C.
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Effect of nucleotides and adenosine on reassociation of phosphorylated and nonphosphorylated R₂ with C

Phosphorylated or nonphosphorylated protein kinase (10 μg) was treated and assayed for reassociation as indicated in the legend to Fig. 1. The above compounds were added (final concentration, 1 mM) after the initial incubation with cAMP. Samples of phosphorylated and nonphosphorylated protein kinase were incubated with cyclic nucleotide phosphodiesterase for 15 and 10 min, respectively.

| Addition | Nonphosphorylated R₂ | Phosphorylated R₂ |
|----------|-----------------------|------------------|
| None     | 65.97                 | 69.36            |
| ATP      | 27.48                 | 23.1             |
| ADP      | 29.61                 | 28.75            |
| AMP      | 35.92                 | 30.67            |
| Adenosine| 29.88                 | 20.14            |
| GTP      | 47.63                 | 40.03            |
| ITP      | 64.46                 | 55.75            |
| CTP      | 67.1                  | 58.94            |
| TTP      | 61.8                  | 60.98            |
| UTP      | 62.66                 | 61.88            |

Table II

Effect of nucleotides and adenosine on protein kinase activity

Protein kinase (0.25 μg) or purified catalytic subunit (0.64 μg) was incubated for 5 min at 4°C in 20 μl of 50 mM potassium phosphate buffer, pH 7.1, containing 4 mM 2-mercaptoethanol and, in the case of the holoenzyme, 20 μM cAMP. Subsequently, the mixture was adjusted to 50 μl by adding the above compounds so that their final concentration in the protein kinase assay (200 μl) was 1 mM. The reaction was initiated by adding the remaining protein kinase assay reaction mixture (see "Materials and Methods") in a volume of 150 μl. Tubes were incubated 2 min at 30°C. One milliunit (mU) of activity is equal to 1 pmol 32P transferred to protamine/min.

| Addition | Holoenzyme | Catalytic Subunit |
|----------|------------|-------------------|
| Activity | Inhibition | Activity | Inhibition |
| milliunits | % | milliunits | % |
| None     | 168.91     | 850.23         |
| ADP      | 9.35       | 94.36          | 48.67    | 94.27 |
| AMP      | 126.64     | 23.86          | 659.94   | 22.38 |
| Adenosine| 36.55      | 77.79          | 150.25   | 82.22 |
| GTP      | 160.87     | 849.73         |
| ITP      | 171.17     | 852.23         |
| TTP      | 176.98     | 849.09         |
| CTP      | 165.51     | 853.02         |
| UTP      | 164.92     | 862.12         |

Despite the initial high concentration of cAMP used in our experiments, the presence of a very active cyclic nucleotide phosphodiesterase enabled us to follow the extent of reassociation of the cAMP-disassociated subunits of protein kinase over a wide range of cAMP concentrations including those believed to be physiological (0.1 to 10 μM (29)). In these studies, total cAMP was measured; the amount of CAMP bound to R₂ was not independently assessed. The following conclusions emerged from these experiments. First, as reported before for cAMP-dependent protein kinases in general (30), the concentration of cAMP appears to be an important factor in the reassociation of both phosphorylated and nonphosphorylated subunits of protein kinase (Figs. 1 and 5). Second, in the presence of cAMP, R₂C₂ accumulates more rapidly than phosphorylated R₂ to achieve the same degree of reassociation lowered 10-fold (Fig. 1, time period 10 to 15 min) for the phosphorylated R₂ has not significantly recombined with C.

The above compounds were added (final concentration, 1 mM) after the initial incubation with cAMP. Samples of phosphorylated and nonphosphorylated protein kinase were incubated with cyclic nucleotide phosphodiesterase for 15 and 10 min, respectively.

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| CTP      | 67.1                  | 58.94            |
| TTP      | 61.8                  | 60.98            |
| UTP      | 62.66                 | 61.88            |

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This analysis does not define the contribution of the various steps requisite for reassociation under the complex conditions of the experiments. It quantitates the amount of phosphorylated and nonphosphorylated holoenzyme formed under such conditions.
The difference in reassociation of the two forms of R_2 was confirmed by analysis of the products of reassociation (Fig. 2) in polyacrylamide gels. These experiments suggest that the reassociation of protein kinase subunits involves the formation of a trimer (R,C) prior to formation of the holoenzyme tetramer. Designation of the R,C structure was based upon the findings that it possessed both regulatory and catalytic subunits, a ratio of R to C activities twice that observed in the holoenzyme (H,R,C) and the predicted molecular weight (137,000).

The inhibitory effect of ATP on reassociation of R_2 with C is worth noting. In the presence of 1 mM ATP and physiological concentrations of cAMP (2 to 20 μM), R_2, unlike phosphorylated R_2, exhibited substantial reassociation and regeneration of the inactive holoenzyme (8, 31). The inhibitory effect of ATP on the reassociation of both forms of protein kinase accentuated the differences between the interactions of R_2 and phosphorylated R_2 with C. The effect of ATP was unrelated to its ability to serve as a phosphate donor for self-phosphorylation of protein kinase since it occurred in the absence of Mg_2+ under conditions which do not allow the phosphotransferase reaction to take place. With the exception of GTP, the effect was specific for adenine-containing molecules. The binding of cAMP, at equilibrium, to either form of R_2 was not affected by ATP. On the other hand, there was a correlation between ability to retard the onset of reassociation and ability to inhibit the catalytic activity of protein kinase. Since the most potent inhibitors, ADP and adenosine, were competitive inhibitors of ATP in the phosphotransferase reaction, it is possible that they can bind to an ATP-specific site on C even in the absence of divalent cations. Haddox et al. (32) and Hofmann et al. (12) demonstrated high affinity ATP-Mg_2+ binding to the Type I protein kinase of skeletal muscle. However, Hofmann et al. (12) were unable to demonstrate that these kinds of sites in the enzyme used here, Type II protein kinase from bovine cardiac muscle. Although the mechanism by which ATP limits the extent of reassociation is unknown, it is likely that at physiological concentrations of ATP (1), in vitro, the holoenzyme is phosphorylated. If the kinase is, in fact, localized in the cell, it may be only partially dissociated by physiological elevations of cAMP. The ability of cAMP to keep the enzyme activated could be potentiated by ATP. In order to inactivate protein kinase, i.e., to permit reassociation to occur in the presence of ATP, the concentration of cAMP would have to fall dramatically so that C could reassociate with phosphorylated R_2. Alternatively, dephosphorylation of R_2 would enable reassociation to take place in the absence of a change in the concentration of cAMP. The discovery of a cardiac muscle phosphoprotein phosphatase that acts on the isolated phosphorylated R_2 rather than the phosphorylated holoenzyme (9, 33) makes this latter mechanism plausible.

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