Dissociation and Reassociation of the Phosphorylated and Nonphosphorylated Forms of Adenosine 3':5'-Monophosphate-dependent Protein Kinase from Bovine Cardiac Muscle

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Adenosine 3':5'-monophosphate (cyclic AMP)-dependent protein kinase from bovine heart muscle catalyzes the phosphorylation of its regulatory, cyclic AMP-binding subunit. Phosphorylation enhances net dissociation of the enzyme by cyclic AMP. Chromatography on a-aminohexyl-agarose was used to study the effects of phosphorylation on cyclic AMP binding and subunit dissociation and reassociation. This method permitted rapid separation of the catalytic subunit from the cyclic AMP-binding protein and holoenzyme. Phospho- and dephosphoprotein kinases were found to dissociate to the same extent at any given concentration of cyclic AMP and completely at saturation. At equilibrium, the amount of cyclic AMP bound was the same for both forms of enzyme and was directly proportional to the degree of dissociation of the holoenzyme.

In the absence of cyclic AMP, phospho- and dephospho-cyclic AMP-binding proteins reassociated completely with the catalytic subunit. However, the rate of reassociation of the dephospho-cyclic AMP-binding protein was at least 5 times greater than the phospho-cyclic AMP-binding protein. Retardation of reassociation was directly proportional to the extent of phosphorylation. We conclude that the degree to which the cyclic AMP-binding protein is phosphorylated markedly affects its intrinsic ability to combine with the catalytic subunit to regenerate the inactive cyclic nucleotide-dependent kinase and that the state of phosphorylation of this subunit may be important in determining the proportion of dissociated (active) and reassociated (inactive) protein kinase at any given time.

Adenosine 3':5'-monophosphate activates the principal soluble bovine heart cyclic AMP-dependent protein kinase by dissociating it into a cyclic AMP-binding protein dimer (R) and cyclic AMP-independent catalytic (C) subunits (1, 2). Purified holoenzyme catalyzes the transfer of $^{32}$P from $[^{\gamma-32\text{P}}]\text{ATP}$ to 2 seryl residues in the subunits of R (3). This phosphorylation enhances net dissociation of protein kinase by cyclic AMP. The phosphate in R can be removed either by reversal of the phosphotransferase reaction (4) or by the action of a cardiac muscle phosphoprotein phosphatase which acts on the dissociated phospho-R (5, 6). In order to understand the mechanism by which phosphorylation influences the activity of protein kinase, we have monitored the dissociation of protein kinase by cyclic AMP and the reassociation of its isolated subunits in the absence of cyclic nucleotides.

**EXPERIMENTAL PROCEDURES**

**Materials**

$[^{\gamma-32\text{P}}]\text{ATP} (> 10 \text{Ci/mmol})$ was purchased from Amersham/Searle; cyclic [3H]AMP (50 Ci/mmol) and Omnifluor were obtained from New England Nuclear Corp.; ATP, cyclic GMP, and cyclic AMP were from Sigma Chemical Co.; protamine sulfate was from Eli Lilly. The aminoalkyl-agarose derivatives were purchased from Miles-Yeda Laboratories.

**Methods**

**Protein Kinase Assay**—Protein kinase was assayed by a modification of a previously described procedure (7). The reaction mixture (200 µl) contained 20 mM potassium phosphate buffer, pH 7.0, 10 mM MgSO$_4$, 50 µM $[^{\gamma-32\text{P}}]\text{ATP}$ (15 to 20 cpm/pmol), 10 mM dithiothreitol, 0.25 mg of protamine sulfate, 0.5 mg of bovine serum albumin, and, where indicated, 2 µM cyclic AMP. Incubations were for 0 or 10 min at 37°C.

**Cyclic AMP-binding Assay**—Cyclic AMP binding was assayed...
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according to Gilman (8) except that 0.05 M potassium phosphate, pH 7.0, was substituted for 0.05 M sodium acetate buffer, pH 4.

Preparation of Protein Kinase—Protein kinase was isolated from bovine cardiac muscle (7). The purified enzyme had a specific activity of 600 to 700 nmoles of ATP transferred to protamine/min/mg of protein and bound 2 mol of cyclic AMP/mole of holoenzyme. It was estimated to be at least 90% pure by polyacrylamide gel electrophoresis in the absence (10) or presence (11) of sodium dodecylsulfate and did not contain detectable cyclic AMP phosphodiesterase activity. The holoenzyme has a molecular weight of 174,000 and contains two catalytic subunits (molecular weight $40,000$ each) and one cyclic AMP-binding protein dimer (molecular weight $98,000$) (12).

Phosphorylation of Protein Kinase—Autophosphorylation was carried out by the procedure of Erlichman et al. (3). The reaction mixture (100 $\mu$l) was incubated at $22^\circ$ for 20 min and contained 100 $\mu$g of purified protein kinase, 50 mM potassium phosphate buffer, pH 7.0, 20 mM MgSO$_4$, and 0.2 mM [$\gamma$-$^3$P]ATP (400 cpm/pmol). For assaying the extent of phosphorylation and cyclic AMP binding, an aliquot of 2 $\mu$l was diluted in 200 $\mu$l of the potassium phosphate buffer, containing 1 mg/ml of bovine serum albumin and 2 x $10^{-5}$ M cyclic [3H]AMP (1800 cpm/pmol). Following additional incubation for 20 min at $4^\circ$, the reaction was terminated by the addition of 1 ml of 50 mM potassium phosphate buffer, pH 6.0, and then poured onto a Millipore filter. The filter, which quantitatively retained R, was washed once with 10 ml of the same buffer and assayed for $^{32}$P and [3H] in coinubulation fluid containing 4 $\mu$g of Omnifloor/filter of toluene. Phosphorylation was expressed as the ratio of moles of $^3$P incorporated per mole of cyclic [PH]AMP bound at saturation. The ratio varied between 0.00 and 0.95.

Purified, unphosphorylated enzyme (5) was treated in the same way as the enzyme undergoing autophosphorylation 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 hours. In preparing isolated subunits for use in the experiments on reassociation, the phosphorylated and unphosphorylated forms of protein kinase were not dialyzed prior to dissociation.

RESULTS

Binding of Protein Kinase to Aminoalkyl-Agarose—A series of columns (13 x 69 mm) containing 2 ml of a homologous series of $\omega$-aminoalkyl-agarose (13) with alky chains 2 (C$_2$), 4 (C$_4$), 6 (C$_6$), 8 (C$_8$), and 10 (C$_{10}$) carbon atoms long were equilibrated at $22^\circ$ with 50 mM potassium phosphate buffer, pH 7.0, containing 4 mM 2-mercaptoethanol. The $10,000 \times g$ supernatant fluid from a cardiac muscle homogenate (1 mg/200 $\mu$l) was applied to each column and subsequently washed with 6 ml of the potassium phosphate buffer. Three fractions of 2 ml each were collected and assayed for protein kinase activity and absorbance at 280 nm. As shown in Fig. 1, the enzyme was completely adsorbed to columns with resin arm lengths of C$_6$ or longer. Both crude and purified protein kinase behaved in the same fashion. Purified protein kinase applied to C$_4$ was eluted with a NaCl gradient (0 to 0.5 M) at a conductivity corresponding to 0.17 M NaCl (Fig. 2). Both phospho and dephospho forms of protein kinase bound to the resin in the same way and were eluted at the same concentration of NaCl. When purified phospho- or dephospho-holoenzyme was applied to a similar column pre-equilibrated with cyclic [PH]AMP (Fig. 3), the enzyme dissociated. The catalytic subunit did not adsorb to the resin, whereas the cyclic nucleotide binding protein (R) was adsorbed under these conditions and could be eluted with 0.20 M NaCl. This resin provided a convenient method for separating the three molecular species of protein kinase (R, C, and holoenzyme) and, as will be described, could be used not only for preparative purposes but for rapid analysis of reassociation and dissociation.

Isolation of Subunits—Cyclic GMP was used to dissociate protein kinase since it, unlike cyclic AMP, can be easily removed from dissociated R by dialysis. For resolving the subunits after dissociation, we used $\omega$-aminoethyl-agarose batchwise as follows: 100 $\mu$g of either phosphorylated or unphosphorylated protein kinase were added to 50 mM potassium phosphate buffer, pH 7.0, 4 mM 2-mercaptoethanol, 75 mM NaCl, 2.5 mg of bovine serum albumin, and 4 mM cyclic GMP (bovine serum albumin was required to preserve the activity of both subunits after dissociation) in a final volume of 1 ml. After 1 min at $4^\circ$, 100 $\mu$l of settled $\omega$-aminoethyl-agarose equilibrated in the same cyclic GMP-containing buffer were added and stirred at $4^\circ$ for 15 min. The agarose was then removed by centrifugation at 3000 x g for 5 min. The supernatant fluid contained the catalytic subunit. The pellet containing bound R was washed once with 4 mM cyclic GMP in the same buffer and then four times with buffer alone. The supernatant fluid obtained after the initial dissociation with cyclic GMP and the first wash was pooled and designated S1. The cyclic nucleotide-binding protein (R) was then eluted by stirring the resin for 10 min at $4^\circ$ with 1.5 ml of 50 mM potassium phosphate buffer, pH 7.0, 0.4 M NaCl, and 2.5 mg/ml of bovine serum albumin. The resin was removed by centrifugation and the supernatant fluid containing R termed S2. Both S1 and S2 were concentrated by dialysis against solid Sephadex G-200 to a final volume of approximately 200 $\mu$l each. The fractions were next 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 hours and assayed for protein kinase activity in the presence and absence of cyclic AMP and for cyclic AMP binding. These assays were used to estimate the concentration of subunits as well as their purity. Since the specific cyclic AMP binding and catalytic activities of the initial holoenzyme could be accurately measured, the concentrations of subunits were calculated on the basis of catalytic activity (for C) or cyclic AMP-binding activity and $^{32}$P incorporation (for R). The actual protein content of the subunit preparations was too low to be accurately assayed. The recoveries for both proteins were 75% to 85% and the contamination of one subunit by the other was generally less than 1%. Equimolar amounts of C and R refer to concentrations of peptide chains, i.e., 2 mol of C are defined as equivalent to 1 mol of R dimer.

Assay for Dissociation of Protein Kinase by Cyclic AMP—The extent of dissociation of the holoenzyme at any given concentration of cyclic AMP was assayed by determining the amount of C remaining in the supernatant fluid after adsorption of the enzyme by $\omega$-aminoethyl-agarose (Fig. 4). Using cyclic [PH]AMP for dissociation, one could determine the amount of cyclic nucleotide bound to R by subsequent filtration on Millipore filters (Fig. 5) and correlate binding of cyclic AMP and dissociation of protein kinase (Fig. 6).

Assay for Reassociation of Isolated Subunits of Protein Kinase—Minicolumns (0.6 x 2 cm), containing 30 $\mu$l of $\omega$-aminoethyl-agarose resin pre-equilibrated with 50 mM potassium phosphate buffer, pH 7.0, 4 mM 2-mercaptoethanol, and 75 mM NaCl at $4^\circ$, were used to separate C from both reconstituted holoenzyme and R within 30 s from the onset of subunit mixing (see Fig. 7). Although this procedure did not completely resolve the holoenzyme from the regulatory subunit, reassociation could be accurately assessed by measuring
or 4", using minicolumns that separated C from R and one of which is presented in Fig. 6.

AMP (0.4 to 1.0 PM) the columns was 86 to 95% (see Table I). The was a direct correlation between the amount of cyclic nucleo-

physiological range (14). The rate of dissociation of both
both phospho and dephospho forms of protein kinase. There
incubation with the holoenzyme was found to be identical for

Results (4) the amount of cyclic ['H]AMP bound to R after

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Distribution of Phospho- and Dephospho-holoenzymes by Cyclic AMP—When phospho- and dephosphoprotein kinases were incubated in the presence of increasing concentrations of cyclic AMP, both dissociated to the same extent (Figs. 3 and 5) and completely at saturation. The similarities in the responses of both enzymes were evident at several concentrations of protein, including one (0.1 PM) considered to be within the physiological range (14). The rate of dissociation of both phospho- and dephosphoprotein kinase (0.05 PM) by cyclic AMP (0.4 to 1.0 PM) was too rapid to be measured at either 22° or 4°, using minicolumns that separated C from R and holoenzyme in less than 1 min.

Binding of Cyclic AMP and Dissociation of Phospho- and Dephosphoprotein Kinases—In agreement with previous results (4), the amount of cyclic ['H]AMP bound to R after incubation with the holoenzyme was found to be identical for both phospho and dephospho forms of protein kinase. There was a direct correlation between the amount of cyclic nucleotide bound and the extent of dissociation (Fig. 6) (15). The same results were observed at two different concentrations of phospho- and dephospho-holoenzymes (0.03 and 0.14 PM), only one of which is presented in Fig. 6.

Rates of Reassociation of Phospho-R and Dephospho-R with C—When phospho-R or R was mixed with equimolar amounts of C, complete reassociation occurred in 0 min at 4°. However, the rates of reassociation were different (Fig. 8). Dephospho-R reassociated at least 5 times more rapidly than its phosphorylated counterpart. Five-fold may, in fact, be an underestimate of this difference since dephospho-R and C reassociated too quickly to accurately measure the half-time of reassociation. We concluded that it is the phosphorylation of R which reduces the rate of reassociation with C rather than some other consequence of treating the enzyme with Mg*+ATP for the following reasons: (a) all of the *P present in phospho-R is covalently bound to R (1) and the *P retained on Millipore filters is completely trichloroacetic acid precipitable; (b) both R and phospho-R were obtained by similar treatment of the holoenzyme except that for the former either ATP was omitted (β, γ-methylene)adenosine 5'-triphosphate substituted for ATP prior to the dissociation procedure, (c) both R and phospho-R bound the same amount of cyclic ['H]AMP at each concentration of nucleotide; (d) R alone, in the absence of C, did not bind γ,*PATP and no evidence was obtained for ['H]ATP binding in the presence or absence of C; and finally (e) when R and phospho-R were mixed in different proportions and then added to C, inhibition of the rate of reassociation was directly related to the proportion of phosphorylated R in the mixture (Fig. 9).

Discussion

We previously reported that phosphorylation of bovine heart protein kinase facilitates its net dissociation by cyclic AMP (3). Since these studies were performed under conditions in which both dissociation and reassociation of subunits could have occurred, we elected to study the effect of phosphoryla-

Dephospho-R reassociated faster than phospho-R to regenerate the cyclic AMP-dependent holoenzyme. In the cyclic AMP-binding studies reported here as well as in studies in which cyclic AMP binding was studied by equilibrium dialysis (6), little difference was found in the abilities of the phospho- and dephospho-
protein kinases to bind cyclic AMP. Both forms appear to bind cyclic AMP with approximately the same affinity and both are completely dissociated upon binding 2 μM of cyclic AMP/molar of holoenzyme. Hofmann et al. (15) reported that the phosphorylated forms of bovine heart protein kinase have a dissociation constant for cyclic AMP which is 5 times lower than the dephospho-holoenzyme. We do not have a satisfactory explanation for this discrepancy in our findings. The fact that there is a linear relationship between cyclic AMP binding and dissociation of protein kinase suggests that once a molecule of cyclic AMP binds to R or phospho-R, one catalytic subunit dissociates from the corresponding holoenzyme. This is in accord with the proposal of Hofmann et al. (15) for the phosphorylated enzyme. We find, however, that this relationship is true for both forms of the enzyme. The substantial quantitative difference attributable to phosphorylation appears to reside in the ability of the phospho- and dephospho-Rs to reassociate with C. Dephospho-R reassociates at least 5 times more rapidly than phospho-R and, conversely, the degree of phosphorylation of R is directly proportional to retardation of reassociation (Fig. 9). Since (a) autophosphorylation occurs at concentrations of ATP that are several orders of magnitude lower than those that obtain under physiological conditions (1) and (b) cardiac muscle phosphoprotein phosphatase appears to act principally on the dissociated phospho-R rather than the holophosphoprotein kinase (5), it is likely that under most physiological conditions the holoenzyme exists in its phosphorylated form. A comparison of the functional differences is perhaps best made at the level of the dissociated enzyme. Our data are most consistent with a model in which the holoenzyme exists principally as a phosphoenzyme. When intracellular levels of cyclic AMP rise, the enzyme binds cyclic AMP and dissociates, yielding phospho-R and C. When cyclic AMP levels fall, phospho-R may reassociate with C to reconstitute the phospho-holoenzyme directly. Alternatively, the phospho-R can be dephosphorylated by phosphoprotein phosphatase, greatly facilitating its capacity to reassociate with C and generate the inactive holoenzyme. The dephospho-holoenzyme would then rapidly self-phosphorylate (4) by an intramolecular reaction to form phospho-holoenzyme. In this pathway, unlike the first, the phosphoprotein kinase would exist as an intermediate in the formation of phosphoprotein kinase from its subunits. If modulation of activity by phosphorylation occurs principally at the level of the dissociated subunits of protein kinase, regulation of dephosphorylation of R may be important in determining the proportion of dissociated (active) and reassociated (inactive) protein kinase at any given time.

Certain cyclic AMP-dependent protein kinases such as the type I protein kinase from rabbit skeletal muscle are not subject to autophosphorylation (15) and it may be that the types I and II protein kinases initially described by Corbin et al. (16) in a variety of tissues may differ fundamentally in this property. The physiological roles of these two kinds of cyclic AMP-dependent protein kinases may be different.
and cyclic AMP binding protein on a minicolumn of ω-aminohexyl-agarose. This figure is a composite of three experiments (A to C) performed on separate but identical columns. (A) Puriﬁed catalytic subunit (0.03 μM or 70 units/20 μl of 50 mM potassium phosphate buffer, pH 7.0, containing 4 mM 2-mercaptoethanol and 75 mM NaCl) was applied to a column (5 × 20 mm) containing 150 μl of settled ω-aminohexyl-agarose equilibrated at 4°C with the buffer indicated above and washed ﬁrst with the same buffer and subsequently with the indicated (ﬁnal) concentration of NaCl in that buffer. Each fraction was 300 μl. O---O, Protein kinase activity assayed in the presence of 1 μM cyclic AMP; □---□, protein kinase activity assayed in the absence of cyclic AMP. (B) R or phospho-R (0.03 μM) was mixed with equimolar C in 20 μl of the same buffer, incubated at 4°C for 15 min by which time reassociation was complete, and processed through the columns as described for the previous experiment. ●---●, Protein kinase activity assayed in the presence of 1 μM cyclic AMP; ■---■, protein kinase activity assayed in the absence of cyclic AMP. (C) R or phospho-R (0.03 μM) was diluted in 20 μl of the same buffer containing 0.03 μM cyclic [3H]AMP (30,000 cpm/pmol) and applied to a column after incubation at 4°C for 10 min. Δ- - Δ, Cyclic [3H]AMP bound to R in 300 μl of sample (see “Methods”). The recoveries of catalytic and cyclic AMP-binding activities in these experiments were 90 to 95% and 78 to 89%, respectively.

FIG. 7 (left). Resolution of the catalytic subunit from holoenzyme and cyclic AMP binding protein on a minicolumn of ω-aminohexyl-agarose. This figure is a composite of three experiments (A to C) performed on separate but identical columns. (A) Puriﬁed catalytic subunit (0.03 μM or 70 units/20 μl of 50 mM potassium phosphate buffer, pH 7.0, containing 4 mM 2-mercaptoethanol and 75 mM NaCl) was applied to a column (5 × 20 mm) containing 150 μl of settled ω-aminohexyl-agarose equilibrated at 4°C with the buffer indicated above and washed ﬁrst with the same buffer and subsequently with the indicated (ﬁnal) concentration of NaCl in that buffer. Each fraction was 300 μl. O---O, Protein kinase activity assayed in the presence of 1 μM cyclic AMP; □---□, protein kinase activity assayed in the absence of cyclic AMP. (B) R or phospho-R (0.03 μM) was mixed with equimolar C in 20 μl of the same buffer, incubated at 4°C for 15 min by which time reassociation was complete, and processed through the columns as described for the previous experiment. ●---●, Protein kinase activity assayed in the presence of 1 μM cyclic AMP; ■---■, protein kinase activity assayed in the absence of cyclic AMP. (C) R or phospho-R (0.03 μM) was diluted in 20 μl of the same buffer containing 0.03 μM cyclic [3H]AMP (30,000 cpm/pmol) and applied to a column after incubation at 4°C for 10 min. Δ- - Δ, Cyclic [3H]AMP bound to R in 300 μl of sample (see “Methods”). The recoveries of catalytic and cyclic AMP-binding activities in these experiments were 90 to 95% and 78 to 89%, respectively.

Fig. 8 (center). Effect of phosphorylation on subunit reassociation. Equimolar amounts of C and R (phospho or dephospho) were mixed at a ﬁnal concentration of 0.03 μM in 20 μl of 50 mM potassium phosphate, pH 7.0, containing 4 mM 2-mercaptoethanol/75 mM NaCl/1 mg/ml of bovine serum albumin and preincubated for different times at 4°C. Samples were then processed as described in Fig. 7. The indicated times refer to the time of preincubation plus the 30 s required to separate C from holoenzyme and R. Reassociation was quantitated by the per cent of cyclic AMP-dependent protein kinase activity eluted with 0.20 M NaCl (see Fig. 7) with respect to the total protein kinase activity eluted from the column (see Table I). The values obtained were then compared with the controls discussed under “Results” (see “Assay for Reassociation of Isolated Subunits of Protein Kinase”). ○---O, R + C; O---O, phospho-R + C.

Fig. 9 (right). Effect of phosphorylation of R on rate of reassociation. Phospho-R and R were mixed in the indicated proportions, added to catalytic subunits at a ﬁnal concentration of 0.03 μM, and then applied immediately to a column of ω-aminohexyl-agarose as described in Fig. 7. The percentage of reassociation was calculated as described in the legend to Fig. 8. The time elapsing between admixture of R and C and elution of C was approximately 30 s for each point.

### TABLE I

**Minicolumn assay for reassociation of isolated subunit of protein kinase**

Puriﬁed catalytic subunits (0.03 μM or 70 units/20 μl of 50 mM potassium phosphate buffer, pH 7.0, containing 4 mM 2-mercaptoethanol and 75 mM NaCl) alone or mixed with equivalent amounts of either phospho or dephospho cyclic AMP binding proteins (0.03 μM) were incubated for 1 min at 4°C and then applied to a minicolumn as described in Fig. 7. Protein kinase activity was assayed in the presence or absence of 1 μM cyclic AMP.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>C</th>
<th>R + C</th>
<th>Phospho-R + C</th>
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<tbody>
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<td></td>
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<td>cAMP</td>
<td>+cAMP</td>
</tr>
<tr>
<td>1</td>
<td>43.34</td>
<td>43.91</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
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AMP-dependent protein kinases which differ signiﬁcantly in their tissue distribution (16) and biochemical (15, 16) and immunological properties (17) have yet to be elucidated. We are currently studying the reassociation of C with R and phospho-R in the presence of different concentrations of cyclic AMP, salt, and protein substrates in order to gain some insight into the physiological effects of phosphorylation and the possible role of dephosphorylation in regulating the net activity of cellular type II protein kinase.

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