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Interconversion of Cyclic Nucleotide-Activated and Cyclic Nucleotide-Independent Forms of a Protein Kinase from Beef Heart

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ABSTRACT **A protein kinase activated by cyclic nucleotides was purified from beef heart. Upon exposure to adenosine 3′:5′-cyclic monophosphate (cyclic AMP) during gel filtration on Sephadex G-200, the protein kinase dissociated into a cyclic nucleotide-independent protein kinase and a cyclic nucleotide-binding protein. A similar or identical cyclic nucleotide-independent protein kinase could be obtained in highly purified form by elution from a DEAE-cellulose column with 10^{-6} M cyclic AMP; the cyclic AMP-binding protein was apparently retained by the resin. The addition of cyclic nucleotide-binding protein to cyclic nucleotide-independent protein kinase resulted in the reappearance of cyclic nucleotide-dependent protein kinase, which could be isolated by filtration on Sephadex G-200 in the absence of cyclic AMP. These results confirm and extend previous suggestions that cyclic nucleotides activate protein kinases by dissociating them from inhibitory, cyclic nucleotide-binding proteins.**

The site of action of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in the chain of events leading to the conversion of muscle glycogen phosphorylase *b* to phosphorylase *a* has been identified as a protein kinase (1, 2) that phosphorylates phosphorylase kinase, as well as a number of other proteins such as casein, protamine, and histone (2). Similar protein kinases, activated by low concentrations of cyclic 3′:5′-mononucleotides, have subsequently been found in a wide variety of animal tissues (3–6) and in bacteria (7). Kuo and Greengard (6) have proposed that the activation of protein kinases by cyclic nucleotides may be the common biochemical mechanism by which these nucleotides exert their varied physiological effects (see ref. 8). This hypothesis is supported by recent reports that some protein kinases possess a greater affinity for cyclic GMP than for cyclic AMP (9), that histones are phosphorylated *in vivo* at sites identical to those phosphorylated by protein kinase *in vitro* (10), and that protein kinases can phosphorylate (and thereby activate) a hormone-sensitive lipase from fat cells (11, 12), as well as microtubular protein from brain (13). Several recent observations pertain to the mechanism by which cyclic nucleotides activate protein kinases. Gill and Garren (14) found that a cyclic AMP-binding protein was enriched in parallel with a protein kinase during purification of the kinase from adrenal cortex. The binding protein was partially dissociated from protein kinase activity by incubation of the enzyme with cyclic AMP. Addition of the binding protein to the assay for protein kinase resulted in inhibition of the kinase activity in the absence of cyclic AMP but not in its presence. This observation suggests that cyclic AMP activates the protein kinase by relieving an inhibition brought about by the association of the protein kinase with a cyclic AMP-binding protein. A similar mechanism for the regulation of protein kinases from skeletal muscle and parotid gland has also been suggested (Z. Sellinger and M. Schramm, personal communication). Tao *et al.* (15) reported that a cyclic AMP-dependent protein kinase from rabbit reticulocytes was dissociated into two subunits in the presence of cyclic AMP. One subunit bound cyclic AMP and lacked catalytic activity, whereas the other subunit possessed kinase activity and could not be activated by cyclic nucleotides. Using sucrose density gradient centrifugation, they estimated that the molecular weights of the catalytic and cyclic AMP-binding moieties were 60,000 and 80,000, respectively. The native, cyclic AMP-dependent kinase had a molecular weight of approximately 140,000. Kumon *et al.* (16) similarly described the progressive inhibition of a cyclic nucleotide-independent protein kinase from rat liver by the addition of a cyclic nucleotide-binding protein. The inhibition was overcome by added cyclic AMP. As with the protein kinase from rabbit reticulocytes, the conversion of a cyclic nucleotide-dependent form of protein kinase to a cyclic nucleotide-independent form of this enzyme was accompanied by its separation from a cyclic nucleotide-binding protein.

In the present communication, we describe the conversion of a cyclic nucleotide-dependent protein kinase to a cyclic nucleotide-independent form and demonstrate that sensitivity of the independent form to stimulation by cyclic nucleotides can be restored upon reassociation of the protein kinase with cyclic nucleotide-binding protein.

MATERIALS AND METHODS

Assay for protein kinase

Protein kinase activity was determined by measuring the incorporation of ^{32}P into protamine (1, 2). The reaction mixture contained, in a final volume of 0.2 ml: 0.05 M potassium phosphate buffer (pH 7.0), 0.01 M $MgSO_4$, 50 μM [γ-^{32}P]-ATP (15,000 cpm/nmol) $\pm$ 1 μM cyclic 3′:5′-nucleotide, 0.25 mg of protamine, 0.5 mg of bovine serum albumin, and enzyme protein. Theophylline (1 mM) and NaF (10 mM) were included in the reaction mixture when crude enzyme (Steps 1–2, Table 1) was assayed. After incubation at 35°C for 5 min, the reactions were stopped by the addition of 0.2 ml of 10% trichloroacetic acid. The protein precipitate was collected by centrifugation, redissolved in 0.1 ml of 1 N NaOH, and then precipitated by the addition of 2 ml of 5% trichloroacetic acid.

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The final precipitate was collected on glass-fiber filters (Whatman GF/C), washed with 20 ml of 5% trichloroacetic acid, and counted in a Nuclear Chicago, low background gas-flow counter.

The assay was linear for 10 min and was proportional to added enzyme up to 100 μg/ml. The ^{32}P incorporated into protamine could be removed by boiling for 10 min in 1 N NaOH.

Protein. Protein concentration was determined by the method of Lowry *et al.* (17), with bovine serum albumin as standard.

Assay for cyclic nucleotide binding

Cyclic nucleotide binding was measured by a modification of the method of Gilman (18). The reaction mixture contained, in a final volume of 0.2 ml: 0.12 M sodium acetate buffer (pH 4.0), 38 μg of protein kinase inhibitor, 0.01 M $MgSO_4$, and 0.5 μCi of [^{3}H]cyclic AMP (12.7 Ci/mmol). The reaction was initiated by the addition of binding protein. Incubations were for 60 min at 4°C, after which the reaction mixtures were diluted to 2 ml with cold 20 mM potassium phosphate, pH 6. The mixtures were then passed through cellulose acetate (Millipore) filters (0.45 μm) and counted in toluene (containing 4 g of Omnifluor/liter) (New England Nuclear) in a Packard Tri-Carb Liquid Scintillation Spectrometer.

Assay for cyclic nucleotide phosphodiesterase activity

Cyclic nucleotide phosphodiesterase activity was assayed by measuring the conversion of [^{3}H]cyclic AMP to [^{3}H]AMP as previously described (19).

Materials

[γ-^{32}P]ATP and Omnifluor were purchased from New England Nuclear, and [^{3}H]cyclic AMP was from Schwarz Bio-Research. Calf-thymus histone, casein, histone-free protamine, and the cyclic 3':5'-nucleotides were obtained from Sigma Chemical Co. Salmon-sperm protamine was purchased from Eli Lilly and Co., Sephadex G-200 from Pharmacia, DEAE-cellulose (DE-52, preswollen) from Whatman, and calcium phosphate gel from Nutritional Biochemicals.

TABLE 1. *Purification of protein kinase*

Step	Volume (ml)	Enzyme units*	Specific activity (units/mg protein)	Recovery (%)
Homogenate	6000	48.60	0.003	100
Ammonium sulfate fraction	360	61.13	0.017	126
Calcium phosphate gel eluate	21	16.08	0.093	33
Sephadex G-200 filtrate	48	11.62	0.326	24
DEAE-cellulose eluate	2	5.60	>10.0†	11

* μmol of ^{32}P incorporated into protamine per 5 min at 35°C in the presence of cyclic AMP (1 μM).

† The protein concentration was too low to be reliably detected by the method employed.

RESULTS

Purification of the protein kinase

Fresh bovine hearts were obtained from a nearby slaughter house, cut into small pieces, and homogenized in a Waring Blendor at top speed for 2 min in 0.25 M sucrose containing 1 mM $MgCl_2$. All the steps in the purification were performed at 4°C. The homogenate was then filtered through cheesecloth

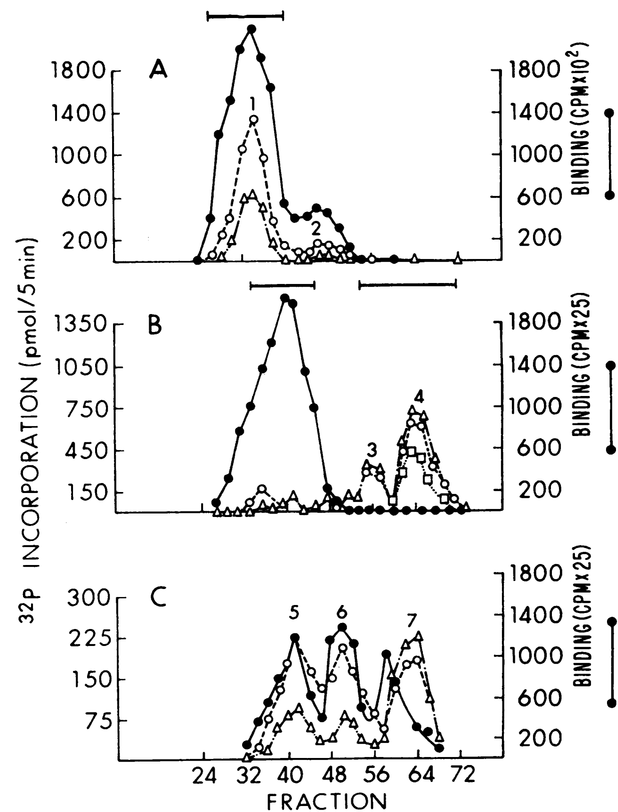

FIG. 1. Pattern of filtration of protein kinase activity and cyclic AMP-binding activity on Sephadex G-200 in the presence and absence of cyclic AMP.

A. Enzyme (40 mg), purified through Step 3 of the purification procedure (see Table 1), was applied to a Sephadex G-200 column. Aliquots (50 μl) of fractions were assayed for kinase activity in the presence and absence of 1 μM cyclic AMP and 100-μl samples were assayed for [^{3}H]cyclic AMP binding. Fractions in Peak 1 (in brackets) were pooled and concentrated. Cyclic AMP (final concentration, 1 μM) was added and the enzyme was applied to a Sephadex G-200 column equilibrated with 1 μM cyclic AMP. The kinase and binding activities that emerged from this column are depicted in *B*.

B. Protein kinase activity was measured immediately; aliquots were dialyzed for 16 hr at 4°C prior to assay for [^{3}H]cyclic AMP-binding. Fractions were collected directly into bovine serum albumin (final concentration: 0.5 mg/ml) in order to preserve enzymic activity. The overall recovery of protein kinase activity was 50%. Bracketed areas were combined, concentrated, dialyzed for 2 hr at 23°C, and again applied to the Sephadex G-200 column, which had been equilibrated with buffer that did not contain cyclic AMP. The kinase and binding activities that emerged from this column are depicted in *C;* about 50% of the protein kinase activity was recovered. (○), Protein kinase activity measured in the presence of 1 μM cyclic AMP; (△), Protein kinase activity measured in the absence of cyclic AMP; (●), [^{3}H]cyclic AMP-binding; (□), Cyclic nucleotide-independent enzyme that had been eluted from DEAE-cellulose by cyclic AMP (Step 5, Table 1).

to remove fat and centrifuged in a Sorvall refrigerated centrifuge for 10 min at 5000 × *g* (Step 1, Table 1). To the supernatant fluid were added solid $(NH_4)_2SO_4$ to 55% saturation and 5 ml of concentrated NH_4OH per liter of enzyme solution. After 1 hr, the suspension was centrifuged for 10 min at 5000 × *g*. The precipitate was resuspended in 0.05 M potassium phosphate buffer (pH 7.0) containing 1 mM mercaptoethanol, and dialyzed against 10 volumes of the same buffer for 16 hr (Step 2, Table 1). Calcium phosphate gel (2 mg/mg protein) was added, with stirring, to the dialyzed enzyme solution. After 30 min, the gel was extensively washed with the same buffer; then the enzyme was eluted with $(NH_4)_2SO_4$ (4 g/100 ml) in the phosphate–mercaptoethanol buffer, in a volume equivalent to that of the enzyme solution originally adsorbed to the gel (Step 3, Table 1). The eluate was concentrated by precipitation with ammonium sulfate (60% saturation) and resuspended in the phosphate–mercaptoethanol buffer. The concentrated solution (5 ml) was applied to a Sephadex G-200 column (2.5 × 85 cm) that had been equilibrated with the same buffer. The column was equipped with upward-flow adaptors; the enzyme was eluted at a rate of 10 ml/hr in 5-ml fractions (Step 4, Table 1). Fractions containing enzymic activity were pooled and applied to a small column of DEAE-cellulose (0.9 × 3 cm) that had been equilibrated with the 0.05 M potassium phosphate buffer, pH 7.0. The column was washed with this buffer (about 100 column volumes) until the eluate was free of material that absorbed at 280 nm. The enzyme was then eluted with 1 μM cyclic AMP in the phosphate buffer. Fractions containing enzyme were pooled and concentrated in an Amicon diaflow concentration cell containing a PM-10 membrane (Step 5, Table 1).

For some experiments, enzyme obtained from Step 4 of the purification procedure was concentrated and again passed through an identical Sephadex G-200 column that had been equilibrated with 1 μM cyclic AMP in phosphate–mercaptoethanol buffer. Both Sephadex G-200 columns were calibrated according to the method of Andrews (20), with Blue Dextran and purified γ-globulin, myoglobin, and bacterial alkaline phosphatase as markers.

Cyclic nucleotide phosphodiesterase was assayed at each stage of the purification procedure. Approximately 98% of the diesterase originally present in the homogenate was removed during elution from the calcium phosphate gel (Step 3, Table 1). There was essentially no diesterase present after the final stage of purification (Step 5, Table 1).

Conversion of the protein kinase from a cyclic nucleotide-dependent to a cyclic nucleotide-independent form

During steps 1–4 of the purification procedure for the protein kinase from beef heart (see Table 1), the enzyme retained its ability to be stimulated by low concentrations of cyclic 3′:5′-nucleotides (see below). Conversion to a cyclic nucleotide-independent form occurred when the enzyme was selectively eluted from DEAE-cellulose by 1 μM cyclic AMP.† Elution was most efficient with cyclic AMP. About 25% of the enzyme could be eluted with 1 μM cyclic GMP; none was eluted with the same concentration of 5′-AMP. Prior to the elution with cyclic AMP, the protein kinase was stimulated 2.5- to 4-fold by cyclic AMP, cyclic GMP, or cyclic IMP. The K_m value for stimulation by cyclic AMP was 6.2×10^{-8} M. After elution from DEAE-cellulose with cyclic AMP, the kinase could not be activated by the addition of cyclic nucleotides, even after it had been subjected to exhaustive dialysis. Indeed, the addition of cyclic AMP generally produced 10–15% inhibition of the protein kinase activity. It is important to note that when cyclic AMP (1 μM) was added to the dependent form of the enzyme, and the enzyme was then dialyzed for 2 hr at room temperature, sensitivity to stimulation by added cyclic nucleotides was retained. This suggested that a fundamental property of the enzyme had been altered during the process of elution by cyclic AMP. Another characteristic of the enzyme after its elution from DEAE-cellulose by cyclic AMP was its relative instability. The cyclic nucleotide-independent enzyme was inactivated by freezing, whereas the dependent form of the enzyme was not. Moreover, the dependent form of the enzyme retained 60% of its activity when heated at 55°C for 10 min, whereas the cyclic nucleotide-independent form was 85% inactivated on heating at 45°C for 10 min.‡ The K_m value for ATP of the dependent enzyme was 13 μM in the absence of cyclic AMP; a similar value (14 μM) was found for the cyclic nucleotide-independent enzyme in the absence of added cyclic nucleotide. The substrate specificity of the two forms of enzyme was also the same. Under the conditions of the assay (see *Methods*), protamine (or histone-free protamine) was phosphorylated 2–3 times as rapidly as was histone, which in turn was phosphorylated at a faster rate than casein. Bovine serum albumin was not significantly phosphorylated. Mg^{++}, Co^{++}, or Mn^{++} satisfied the divalent-metal requirement of both forms of the enzyme, and the pH at which optimal activity was obtained was approximately 7.8 (in Tris·HCl buffer) for both.

Gel filtration of the cyclic nucleotide-dependent and cyclic nucleotide-independent forms of protein kinase

The cyclic nucleotide-dependent kinase from any stage of purification (Steps 1–4, Table 1) emerged as a single peak, corresponding to a molecular weight of about 240,000, from Sephadex G-200. An additional minor peak of cyclic nucleotide-dependent protein kinase activity was also found; it represented about 10% of the total protein kinase activity (Peak 2, Fig. 1*A*). This fraction was discarded.

The cyclic nucleotide-independent kinase was obtained by elution from DEAE-cellulose with cyclic AMP. When subjected to gel filtration on Sephadex G-200, it emerged as a single peak with a molecular weight of about 30,000 (Peak 4, Fig. 1*B*). Cyclic nucleotide-independent protein kinase could also be obtained by passing the major peak of dependent protein kinase eluted from Sephadex G-200 (Step 4, Table 1) through Sephadex G-200 in the presence of 1 μM cyclic AMP. The enzyme recovered from this column emerged in the same elution volume as did the cyclic nucleotide-independent kinase that had been prepared by cyclic AMP elution from DEAE-cellulose (Step 5, Table 1). In addition to this fraction, a second peak containing cyclic nucleotide-independent protein kinase, molecular weight 60,000–70,000, was also consistently observed when this method of preparation was employed (Peak 3, Fig. 1*B*).

† A similar method has also been successfully employed for preparing a cyclic nucleotide-independent protein kinase from frog erythrocytes, using 0.1 μM cyclic AMP as eluent (J. Erlichman and O. M. Rosen, unpublished results).

‡ Equivalent protein concentrations for this experiment (10 mg/ml) were maintained by the addition of bovine serum albumin to the cyclic nucleotide-independent enzyme.

Gel filtration of cyclic nucleotide-binding protein

When the cyclic nucleotide-dependent form of the protein kinase was passed through Sephadex G-200, material capable of binding [^{3}H]cyclic AMP was detected in a peak that coincided with that containing the cyclic nucleotide-dependent protein kinase activity (Fig. 1*A*). A fraction containing the binding protein, unassociated with kinase activity, was obtained by passing the cyclic nucleotide-dependent kinase through a Sephadex G-200 column in the presence of 1 μM cyclic AMP (Fig. 1*B*). Under these conditions, cyclic nucleotide-binding activity appeared in a peak corresponding to a molecular weight of about 160,000. After the individual fractions of this peak were dialyzed for 16 hr at 4°C, binding activity increased about 10-fold. Even this activity, however, was less than that associated with the native, cyclic nucleotide-dependent, protein kinase that had been filtered on Sephadex G-200 in the absence of cyclic AMP. This variation in binding of [^{3}H]cyclic AMP was probably due to incomplete removal of nonradioactive cyclic AMP from the binding protein by dialysis at 4°C. The high affinity of cyclic nucleotide-binding proteins for cyclic nucleotides under these conditions has been commented upon previously (21).

When fractions eluted from DEAE-cellulose with cyclic AMP were assayed for their ability to bind [^{3}H]cyclic AMP, binding activity was not detected, even after exhaustive dialysis of the fractions to diminish possible interference due to the presence of nonradioactive cyclic AMP. It is probable, therefore, that the cyclic nucleotide-binding activity was not eluted from DEAE-cellulose by cyclic AMP.

Conversion of cyclic nucleotide-independent forms of the protein kinase to cyclic nucleotide-dependent forms

The cyclic nucleotide-binding activity and cyclic nucleotide-independent protein kinase activities that had been eluted from a Sephadex G-200 column in the presence of 1 μM cyclic AMP were combined, concentrated, and dialyzed for 2 hr at 23°C. Prior to the addition of binding protein, there were 1.3 $\times 10^6$ units of protein kinase activity, assayed in the presence or absence of cyclic AMP. After the binding protein had been added and the pooled fractions were concentrated and dialyzed, the total activity measured in the absence of cyclic AMP decreased to 0.8 $\times 10^6$ units. Activity was restored to the original value, however, by the addition of 1 μM cyclic AMP. Aliquots of protein kinase that were concentrated and dialyzed in the absence of binding protein exhibited no loss of activity. Thus, the addition of cyclic AMP-binding protein resulted in an inhibition of kinase activity, which was overcome by the addition of cyclic AMP, suggesting that the cyclic nucleotide-independent protein kinase had been reconverted to a cyclic nucleotide-dependent state. This was confirmed by passing the concentrate through Sephadex G-200 in the absence of cyclic AMP. The elution pattern was dramatically altered (Fig. 1*C*). There was a diminution in the cyclic nucleotide-independent form of the protein kinase (Peak 7, Fig. 1*C*, as compared with Peak 4, Fig. 1*B*) and the appearance of two new protein kinase peaks, both of which were stimulated by cyclic AMP and associated with cyclic nucleotide-binding activity. The molecular weights of these cyclic nucleotide-dependent protein kinase activities were about 140,000 and 70,000 (Peaks 5 and 6, Fig. 1*C*). A new peak of cyclic nucleotide-binding activity, unaccompanied by kinase activity, was also apparent. Its molecular weight was about 40,000. The native, cyclic nucleotide-dependent, protein kinase of molecular weight 240,000 was not regenerated under these conditions. Experiments in which the cyclic nucleotide-independent forms of protein kinase were passed through Sephadex G-200 in the absence of cyclic AMP, but without the addition of cyclic nucleotide-binding protein, failed to reveal any conversion to forms that could be activated with cyclic AMP.

CONCLUSIONS

The studies described here demonstrate that cyclic AMP can promote the dissociation of a cyclic nucleotide-dependent protein kinase into a cyclic nucleotide-independent form and a cyclic nucleotide-binding protein. The process of dissociation is at least partially reversible, since cyclic nucleotide-dependent protein kinase activity can be regenerated by combining the binding activity with the cyclic nucleotide-independent protein kinase in the absence of cyclic nucleotides. Thus, the results confirm previously proposed mechanisms for the activation of protein kinases by cyclic nucleotides (14–16). It has been reported that cyclic nucleotide-binding proteins inhibit the activity of cyclic nucleotide-independent kinases and that this inhibition can be overcome by the addition of cyclic AMP (14–16). Although reassociation of cyclic nucleotide-binding protein with cyclic nucleotide-independent kinase can be effected *in vitro*, it is not known how this process occurs *in vivo;* the mechanisms for inhibition of protein kinase activity *in vivo* require further investigation. The observation that cyclic AMP is released from its binding protein much more rapidly at 23–35°C than at 4°C (J. Erlichman, A. H. Hirsch, and O. M. Rosen, unpublished experiments) may explain why a preliminary dialysis at room temperature is effective in permitting reassociation of the binding protein with the cyclic nucleotide-independent protein kinase and suggests that reassociation of the binding protein with the cyclic nucleotide-independent kinase may occur with much greater efficiency *in vivo* than in the laboratory cold room.

Since we have not yet obtained homogeneous preparations of the cyclic nucleotide-binding activity, it may be that factors other than the binding protein are necessary for the formation of the cyclic nucleotide-dependent kinase or for the reformation of the native protein kinase. The concentration of enzyme protein may also be an important factor in determining the molecular form of the cyclic nucleotide-dependent protein kinases. Elucidation of the precise molecular structure of the cyclic nucleotide-dependent kinases must await further purification of their component parts and more rigorous estimation of their molecular weights.

NOTED ADDED IN PROOF

After this manuscript was submitted for publication, Reimann, E. M., C. O. Brostrom, J. D. Corbin, C. A. King, and E. G. Krebs, *Biochem. Biophys. Res. Commun.*, **42**, 187(1971) reported that the protein kinase of rabbit skeletal muscle could, in the presence of cyclic AMP, be dissociated into a cyclic AMP-binding subunit and a cyclic AMP-independent protein kinase subunit.

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