Proc. Nat. Acad. Sci. USA
Vol. 68, No. 10, pp. 2444–2447, October 1971

Interaction of the Subunits of Adenosine 3′:5′-Cyclic Monophosphate-Dependent Protein Kinase of Muscle

(regulatory subunit/catalytic subunit/cAMP· regulatory subunit complex)

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Communicated by Hans Neurath, July 20, 1971

ABSTRACT **Two cAMP-dependent protein kinases purified from rabbit skeletal muscle were shown to bind the same amount of cAMP per unit of enzyme activity at several concentrations of this nucleotide. A preparation containing both of these kinases was separated into catalytic (C) and regulatory (R) subunit fractions in the presence of cAMP, the regulatory subunit being obtained as an R·cAMP complex. Addition of increasing amounts of the R·cAMP complex to the holoenzyme (RC) increased the concentration of cAMP required for half-maximal activity of the enzyme. cAMP was liberated from the R·cAMP complex in the presence of added catalytic subunit in a reaction that was facilitated by Mg^{2+}, ATP, and warming. These findings are presented in support of a model for activation of the protein kinase by cAMP. The possibility that excess regulatory subunit may serve as a sink for intracellular cAMP is also discussed. It is shown that cAMP bound to the R subunit is not a substrate for the cAMP phosphodiesterase.**

The existence of protein kinases that are activated by adenosine 3′:5′-cyclic monophosphate (cAMP) has been documented extensively for various tissues from several species (1–9), and some regulatory functions of cAMP have been shown to be mediated by this type of enzyme (1, 5, 9–12). On the basis of kinetic studies, and in view of the stability properties of the heart-muscle cAMP-dependent protein kinase, it was proposed (6) that the enzyme was made up of regulatory (R) and catalytic (C) subunits, which dissociated in the presence of cAMP:

$$\underset{\text{(inactive complex)}}{C \cdot R} \rightleftarrows \underset{\text{(active enzyme)}}{C} + R \qquad (1)$$

R, the cAMP-binding subunit, was viewed as an inhibitor of the catalytic subunit. Independently, Gill and Garren found that adrenal cortical extracts contained a cAMP-binding protein that was partially separable from the protein kinase activity in their preparations (7). Addition of the binding protein to the protein kinase fraction inhibited its activity when it was tested in the absence of cAMP; and these workers also suggested that cAMP might be causing dissociation and activation of the enzyme. Tao, Salas, and Lipmann (13) showed that a protein kinase from rabbit reticulocytes could be dissociated by cAMP into catalytic and cAMP-binding subunits, and suggested a scheme essentially identical to that of Eq. 1, as did Kumon, Yamamura, and Nishizuka, who worked with the liver enzyme (14). The latter workers obtained complete separation of what they referred to as the R-protein from the catalytic or cAMP-independent subunit. The regulatory subunit (R) was completely separated from the catalytic subunit (C) of the skeletal-muscle enzyme in this laboratory (15).

The present study is concerned with the interactions that occur between cAMP and the isolated R and C subunits of the skeletal-muscle protein kinase. Evidence is presented to show that C causes displacement of cAMP bound to R, in a reaction that is facilitated by Mg^{2+} and ATP. The sensitivity of the reconstituted protein kinase to cAMP depends upon the ratio of R to C. It is proposed that these findings implicate the existence of a dynamic equilibrium among these components:

$$RC + cAMP \rightleftharpoons R \cdot cAMP + C \qquad (2)$$

It follows from this equation that hormonal elevation of the intracellular concentration of cAMP would shift the above equilibrium towards the formation of the active form of protein kinase (C); conversely, depression of the intracellular cAMP concentration would lead to the formation of the repressed form of the enzyme (RC).

MATERIALS AND METHODS

Protein kinase catalytic activity was determined by the incorporation of phosphate from [γ-^{32}P]ATP into casein (16). cAMP and 5′-AMP were identified by descending chromatography, using Whatman No. 3 chromatography paper and a solvent consisting of *n*-butanol–acetone–glacial acetic acid–ammonia (28–30%)–water 70:50:30:5:45. The binding of cAMP by the protein kinase was measured on UM-10 ultrafiltration discs in an eight-chambered ultrafiltration cell purchased from the Metaloglass Co. of Boston, Mass. This device was designed for the rapid measurement of ligand binding to protein by the method of Paulus (17). [8-^{3}H]cAMP (15.7 Ci/mmol) was purchased from Schwarz BioResearch, Orangeburg, N.Y. Tritium was measured in a Packard model 3375 Liquid Scintillation Spectrophotometer.

RESULTS

Binding of cAMP to the protein kinases of rabbit skeletal muscle

Purified protein kinase from rabbit skeletal muscle was prepared by the method of Reimann *et al.* (16) through the DEAE-cellulose step. The first peak of enzyme to be eluted from the column (Peak I) was used exclusively in the following studies, unless noted otherwise. This fraction was further separated into its heavy (6.8 S) and light (5.2 S) protein-

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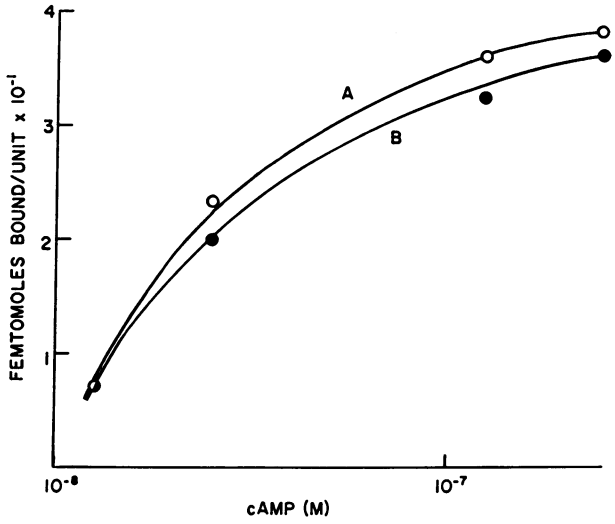

FIG. 1. Binding of cAMP by protein kinases of skeletal muscle. Preparations of the heavy (6.8S) and light (5.2S) protein kinases of muscle were adjusted to the same amount of protein kinase activity, as measured in the presence of excess cAMP, and the binding of [^{3}H]cAMP to enzyme in 100-μl aliquots was determined by the ultrafiltration method. The unit of enzyme activity is defined as the amount of enzyme catalyzing the incorporation of 1 pmole of ^{32}P into casein per min in an activity test with [^{32}P]-ATP (16). *Curve A* is with the 6.8S enzyme and *Curve B* with the 5.2S enzyme. The *ordinate* scale is femtomoles of bound cAMP per unit of enzyme activity ($\times 10^{-1}$).

kinase components by sucrose density gradient centrifugation (16), and the ability of each of these enzymes to bind [^{3}H]-cAMP was tested (Fig. 1). The amount of cAMP bound per unit of enzyme activity for different concentrations of [^{3}H]cAMP was the same for each species of the kinase. The amount of cAMP bound at infinite cAMP concentration, about 39 fmol† per unit of enzyme activity for each enzyme, was determined from reciprocal plots of these data (not illustrated). The heavy and light protein kinases of Peak I have identical C subunits, but differ in the size of their respective R subunits (15). It can now be concluded that each type of R subunit binds the same amount of cAMP per mole, if it is assumed that the molar ratio of R to C is identical for each enzyme. For this conclusion to be valid, it must also be accepted that the catalytic activity of the protein kinases at infinite cAMP concentration is a measure of the amount of C present.

The amount of cAMP bound to the R subunits of the protein kinases from muscle was also determined, incidental to the preparation of the C and R · [^{3}H]cAMP components of the enzyme. For this preparation, Peak I protein kinase was chromatographed on a substrate affinity column prepared from casein–Sepharose 4B in the presence of [^{3}H]cAMP (15). Essentially all of the protein kinase activity, which was now cAMP-independent, was recovered in this procedure; it was completely separated from the cAMP-binding protein. Free [^{3}H]cAMP was removed from this latter fraction (R) with Sephadex G-25, and the bound nucleotide remaining was then determined. The bound [^{3}H]cAMP amounted to 32 fmol of cAMP per unit of enzyme activity applied to the casein–

† 1 fmol (femtomole) = 10^{-15} mol.

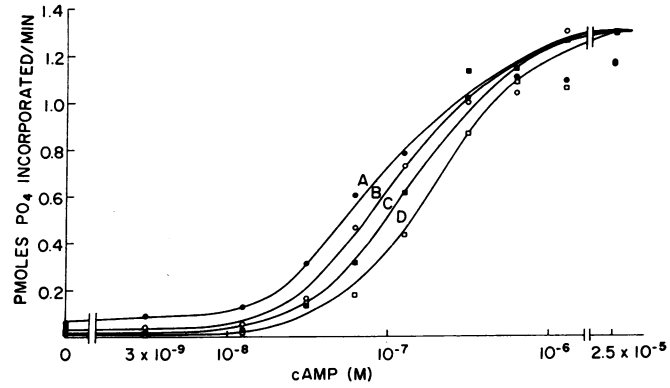

FIG. 2. Effect of R·cAMP on the activity of the protein kinase at different concentrations of cAMP. Reaction mixtures contained the standard protein kinase assay components (all curves), plus purified R·cAMP fraction in *Curves B–D*. The protein kinase used was the 6.8S component obtained from Peak I (See *Methods* and Fig. 1). Ratios of R·cAMP to C were as follows: *Curve A*, 1.0; *Curve B*, 6.0; *Curve C*, 12.0; and *Curve D*, 30.0.

Sepharose column. This value is in close agreement with that determined by the ultrafiltration method used in studying the individual protein kinases present in Peak I (Fig. 1). The calculations also show that the R fraction as it is prepared is, to all intents and purposes, saturated with cAMP, i.e., that it consists of an R · [^{3}H]cAMP complex. Other experiments (not illustrated) also showed that the isolated complex was incapable of binding significant additional amounts of [^{3}H]cAMP.

Increased cAMP requirement by protein kinase in the presence of added R·cAMP

If the mechanism of action of cAMP as an activator of the protein kinase is in accord with the equilibrium shown in Eq. 2, it would be expected that addition of the R · cAMP complex to protein kinase reaction mixtures would increase the requirement for cAMP, assuming that RC is an inactive form of the enzyme. To test this idea, an experiment was designed in the presence of several concentrations of added R · [^{3}H]cAMP (Fig. 2). Since we knew the [^{3}H]cAMP-binding capacity of holoenzyme and the [^{3}H]cAMP content of the added R · cAMP preparation, it was possible to express concentrations in terms of the ratio of total R to total C. It was arbitrarily assumed that the ratio is 1:1 in the protein kinase itself. As this ratio was increased from 1:1 (*Curve A*) to 30:1 (*Curve D*), a 3- to 4-fold increase in the concentration of cAMP was needed to attain half-maximal catalytic activity.

A similar experiment that used isolated R · cAMP and C was not feasible because these subunits require a finite time to recombine (Table 1). Over the period in which the subunits were recombining, catalytic activity in the absence of added cAMP was continually declining. The rate of decline was dependent on the amount of R·cAMP added.

Liberation of [^{3}H]cAMP from the R·[^{3}H]cAMP complex

It was shown (16) that all the bound radioactivity present in a protein kinase preparation to which [^{3}H]cAMP had been added was released as [^{3}H]cAMP when the proteins were denatured by heating or by the use of any of several denaturating agents. This result was confirmed for the tritium bound in the isolated R · [^{3}H]cAMP complex. Various conditions

TABLE 1. *Time dependence for the recombination of R·cAMP and C, as measured by inhibition of catalytic activity*

Incubation time, min	Moles of ^{32}P incorporated	
	− cAMP	+ cAMP
0	12.7	39.6
2	8.0	40.5
5	2.7	39.2
10	1.3	40.8
30	0.8	39.6
40	0.3	40.0

Incubation mixtures at 30°C were prepared containing 0.21 mM nonradioactive ATP; 5 mM magnesium acetate; 1 mg/ml casein; and the R and C fractions, at a ratio of 1.6:1, respectively, in a final volume of 1.2 ml. The C fraction was added at zero time. At intervals aliquots were removed, diluted, and assayed for protein kinase activity for 20 min at 30°C with [^{32}P]ATP, in the presence and absence of cAMP. The carryover of cAMP into the protein kinase reaction mixtures due to bound [^3H]cAMP in the R·cAMP fraction was only 1×10^{-9} M.

were then examined to determine the feasibility of producing R, free of cAMP, but without denaturing this subunit. These experiments were unsuccessful, but did indicate how tightly cAMP is bound in the R·cAMP complex. Gel filtration at pH 7.5 at 4°C in the presence or absence of 1 M NaCl was completely ineffective in removing the bound nucleotide. Incubation at 4°C with either 4 M urea or 1 mM dithiothreitol for 1 hr, or with 0.1% Triton X-100 for 10 min, followed by gel filtration was also ineffective. The bound tritium did not exchange with nonradioactive cAMP (1 mM) during gel filtration at 4°C and was not removed in electrofocusing experiments. The nucleotide was not removed in sucrose density gradient experiments at 20°C. The bulk of the tritium was removed by prolonged dialysis at 25°C against Norite in the presence of 10 mM N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid at pH 7.5, but the recombining capacity of R was proportionately decreased, indicating that denaturation of the protein had occurred.

Since it was known that addition of the R·[^3H]cAMP complex to the C subunit gives rise to a cAMP-dependent protein kinase (15), it seemed likely that [^3H]cAMP would be released during recombination of the isolated subunits if the mechanism in Eq. 2 is correct. That this expected result did occur is shown by the experiments presented in Table 2. Samples of R containing bound [^3H]cAMP were incubated for 5 min at 0°C or 30°C in the presence or absence of added C (plus other additions). Liberated [^3H]cAMP was determined by gel filtration. At 0°C, addition of C alone caused a significant release of [^3H]cAMP. ATP, Mg^{2+}, and the combination of these components facilitated this release. At 30°C there was little, if any, release of [^3H]cAMP without added C, but again there was a marked effect on adding C, ATP and/or Mg^{2+}.

Resistance of cAMP bound to R to attack by diesterase

By the use of a less highly purified R fraction than that used in the experiments we described above, it was possible to study the accessibility of cAMP bound to R to a cAMP phosphodiesterase activity present as a contaminant in the preparation (Table 3). On incubation of this fraction containing the R·[^3H]cAMP complex for 30 min in the presence of

TABLE 2. *Effect of C on the release of [^3H]cAMP from the R·[^3H]cAMP complex*

Addition	Percent of counts released at	
	0°C	30°C
None	0	3
ATP + Mg^{2+}	...	3
C	12	35
C + ATP	36	53
C + Mg^{2+}	55	58
C + ATP + Mg^{2+}†	83	88

Mixtures of 200 μl R fraction containing bound [^3H]cAMP; 40 μl C fraction, where added; 5 mM magnesium acetate, where added; 1 mM ATP, where added; 1 mM EDTA; and 10 mM 2[*N*-morpholino]ethane sulfonic acid (pH 6.5), in a total volume of 250 μl, were incubated at 0°C or 30°C for 5 min and then passed through 0.9 × 60 cm Sephadex G-25 columns equilibrated at 4°C with the nonprotein components of the incubation mixtures over a 2-hr period. Tritium that was excluded (bound cAMP) and that which entered the gels (free cAMP) was analyzed by scintillation counting, and identified by paper chromatography as cAMP.

Mg^{2+}, there was no significant reaction. When the C subunit was added, however, about half the cAMP pressent was converted to 5'-AMP in 30 min. [^3H]cAMP added in excess (over and above that bound as R·cAMP) was attacked without addition of C. In a separate control experiment (not illustrated), it was demonstrated that the C fraction used in this experiment was free of phosphodiesterase.

DISCUSSION

The observations that cAMP is readily removable from the R·cAMP complex in the presence of the catalytic subunit, C, and that cAMP causes the dissociation of RC (13–15) may be taken as evidence for the existence of a dynamic equilibrium between R, C, and cAMP as shown in Eq. 2. In addition, the finding that an increase in the ratio of R to C leads to a requirement for higher concentrations of cAMP to achieve half-maximal catalytic activity (Fig. 2) may be cited as additional support for such an equilibrium. In view of these data, it is apparent that a K_M value cannot be assigned for the interaction of cAMP with protein kinase unless the ratio of R to C is specified. Walsh *et al.* (18) have recently shown that a protein inhibitor of cAMP-dependent protein kinases promotes a 5-fold increase in the binding constant of the enzyme for cAMP; this observation has been used by Gilman (19) as the basis for an assay of cAMP binding by protein kinase. A possible explanation underlying this effect is that the inhibitor combines with free catalytic unit‡ to remove it from participation in the equlibrium expressed by Eq. 1. The equilibrium is thus, in effect, shifted toward the formation of R·cAMP, which binds cAMP tenaciously in the absence of C. The presence of phosphodiesterase activity, on the other hand, would shift this equilibrium toward the formation of RC, through the hydrolysis of cAMP. It has been reported that insulin stimulates the activity of phosphodiesterase in some tissues (20, 21). If the action of insulin were to lower the concentration of cAMP in muscle, as it does in some tissues

‡ Reimann, E. M., unpublished result.

TABLE 3. *Inability of cAMP-phosphodiesterase to catalyze the hydrolysis of [^{3}H]cAMP bound to R*

Addition	Recovery of nucleotides (%) as cAMP	as 5'-AMP
None	98	2
C*	100	0
C	48	52
[^{3}H]cAMP†	42	48

A preparation of R·[^{3}H]cAMP complex containing cAMP phosphodiesterase activity was obtained by separating the subunits of a relatively crude protein-kinase preparation from rabbit skeletal muscle on a casein–Sepharose affinity column. This crude enzyme was prepared by chromatography of muscle extract on a histone–Sepharose 4B affinity column at pH 6.1, immediately after precipitation of impurities at pH 5.6 (16). The enzyme was eluted from the histone column with 1 M NaCl and chromatographed on Sephadex G-100, to give a 30-fold purification from the original extract. For the above experiment, incubation tubes contained the R fraction (1×10^5 cpm of bound [^{3}H]cAMP), 5 mM magnesium acetate, and a preparation of the C fraction (where added). After 30 min of incubation at 30°C, the mixtures were boiled and aliquots were examined by paper chromatography for residual [^{3}H]cAMP and [^{3}H]5'-AMP formed.

* Magnesium acetate omitted.

† Free [^{3}H]cAMP, in an amount five-times greater than [^{3}H]cAMP bound to R, was added to this tube.

(22–24), then the recombination of R and C would be favored in the above equilibrium, i.e., the protein kinase activity would seem more cAMP-dependent. By the same logic, any hormone that affects the concentration of intracellular cAMP would shift this equilibrium accordingly.

The presence of relatively high intracellular concentrations of cAMP in muscle and other tissues is well documented (22, 25), and it has been proposed that the cAMP may be sequestered in some manner (22, 26). The results of Table 3 suggest that cAMP phosphodiesterase may not be able to act on cAMP that is bound to R. A similar conclusion has also been drawn by Odea *et al.*§ from work with a system containing a purified phosphodiesterase and protein kinase from bovine skeletal muscle. Therefore, it is possible that free R could serve as an intracellular reservoir for cAMP. Fairly high cellular concentrations of R relative to C would be necessary if R did, indeed, function in this capacity; experiments are now in progress to estimate the concentrations of each of these subunits in the cell.

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We thank Drs. Donal A. Walsh and Robert R. Traut for helpful suggestions in the preparation of this manuscript. This work was supported by a grant from the American Heart Association. J. D. C. was a recipient of a Research and Development Award of the American Diabetes Association.

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