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cAMP-DEPENDENT PROTEIN KINASE FROM MUCOR ROUXII: PHYSICAL EVIDENCE OF A TERNARY COMPLEX HOLOENZYME-cAMP

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SUMMARY: Gel electrophoresis and sucrose density gradient centrifugation techniques permitted the visualization for the first time of the ternary complex formed by the binding of cAMP to Mucor rouxii cAMP-dependent protein kinase holoenzyme. The addition of 0.5 M NaCl or histone plus ATP-Mg^{++}, together with cAMP, dissociates the holoenzyme into free regulatory (R) and catalytic (C) subunits. At 4°C, cAMP bound to the holoenzyme is readily exchangeable with unlabeled cAMP (half life 2.5 min), while the nucleotide bound to the R subunit has a very slow exchange rate (half life 210 min). The amount of cAMP bound to R subunit is approximately twice the amount bound to holoenzyme at saturation.

cAMP activated protein kinases seem to mediate the effect of cAMP on many eucaryotic processes (1). These enzymes are known to be formed of two subunit types (regulatory R and catalytic C) that interact with each other and with cAMP as follows (2,3):

$$R_2C_2 + 4\ cAMP \rightleftharpoons R_2cAMP_4 + 2\ C$$

inactive holoenzyme — active

Although the initial reactants and final products of the overall reaction have been demonstrated, the detailed mechanism for the process has not been firmly established. Several authors have presented kinetic evidence strongly supporting the formation of a ternary complex holoenzyme-cAMP as an obligatory step in the dissociation process (4-11), but such a complex has never been isolated.

In the present study we isolate a ternary complex, formed by the binding of cAMP to cAMP-dependent protein kinase from Mucor rouxii. We have already reported the partial purification and characterization of this enzyme isolated from extracts of the fungus (12, 13)

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MATERIALS AND METHODS

Unless otherwise indicated, experimental details were as described in preceding papers (12, 14) or as provided in the text and figure legends.

Yeast cells of Mucor rouxii (NRRL 1894) were used throughout. [^{3}H]cAMP (specific activity 38 Ci/mmol) and [^{32}P]ortophosphate for the preparation of [γ-^{32}P] ATP (15) were obtained from the Radiochemical Centre, Amersham.

Protein kinase assay. The enzymatic activity was assayed as previously described (13) using 1.5 mg/ml histone (Type II S, Sigma) or 0.25 mg/ml kemptide (Sigma) as substrates. When kemptide was used as substrate, the [^{32}P]phosphopeptide was measured as described by Glass et al (16). At these saturating concentrations, kemptide was approximately 30 times better substrate than histone. One unit of protein kinase activity was defined as the amount catalyzing the transfer of one pmol of [^{32}P] into histone per minute under the conditions of the standard assay.

cAMP-binding assay. [^{3}H]cAMP-binding was assayed as previously described (13) by the membrane filtration technique(17) using Schleicher & Schüll BA-85 membrane filters.

Polyacrylamide gel electrophoresis. Electrophoresis in 5% polyacrylamide gels was performed at 4°C according to the procedure of Davis (18). Following electrophoresis, some gels were stained with Coomassie Brilliant Blue. Other gels were cut into 2 mm slices and processed as described in the legend of Fig. 3.

Protein assay. Protein was measured by the method of Bradford (19) using bovine serum albumin as standard.

cAMP-dependent protein kinase preparation. The enzyme was prepared as previously described (13) up to the DEAE-cellulose step, except that the buffer used throughout the whole preparation was 20 mM Tris-HCl pH 7.4, 4 mM EGTA, 4 mM EDTA, 2 mM mercaptoethanol, 3 mM benzamidine (Buffer I). Aliquots of 300 μl of the preparation (5 mg protein/ml) were loaded onto six 4.5 ml linear sucrose gradients (5-20%) in buffer I and centrifuged for 16 hours at 40,000 rpm in a SW 55 Ti rotor. 25 fractions of each gradient were collected. The peak of protein kinase activity sedimenting at 8.8 S was concentrated through DEAE-cellulose, and stored in aliquots at -20°C. This preparation, analyzed by sucrose gradient centrifugation, did not contain detectable amounts of free catalytic or regulatory subunits, nor cAMP phosphodiesterase activity. This final preparation had a specific protein kinase activity of approximately 1,700 units/mg and the stimulation by 1 μM cAMP ranged from 7-11 fold.

Preparation of the regulatory subunit - [^{3}H]cAMP complex. The R-[^{3}H]cAMP complex was prepared according to the following procedure. Fractions containing [^{3}H]cAMP-binding activity from six gradients similar to that of Fig. 1C were pooled, dialyzed and concentrated through DEAE-cellulose. Two aliquots of the isolated complex were incubated at 4°C with or without 300 nM [^{3}H]cAMP. The [^{3}H]cAMP bound in both cases was similar, indicating that the complex remained saturated.

RESULTS AND DISCUSSION

Visualization of the ternary complex on sucrose gradient centrifugation

As previously reported (12, 13) the protein kinase of Mucor rouxii, in a wide range of enzyme concentration (50-2000 units/ml) was not completely dissociated by preincubation with micromolar concentrations of cAMP, unless 0.5M NaCl was included in the mixture.

TABLE I: Binding of cAMP to protein kinase under dissociating and nondissociating conditions

[^{3}H]cAMP μM	NaCl 0.5 M	[^{3}H]cAMP bound (cpm)
0.025	-	3000
0.050	-	4800
0.140	-	6500
0.280	-	6900
2.8	+	14000

10 units of the holoenzyme preparation were incubated 60 min at 4°C with the additions described in the Table. [^{3}H]cAMP bound was measured as described in Materials and Methods.
(The values shown are average of triplicates in single experiments).

Sucrose gradient centrifugation was used to further investigate the interaction of holoenzyme with cAMP. Fig. 1A shows the profile of sedimentation of catalytic and binding activities of a control holoenzyme. When the enzyme was preincubated with saturating concentrations of [^{3}H]cAMP (see Table I) and [^{3}H]cAMP (0.13 μM) was mantained in the gradient, the profile depicted in Fig. 1B was obtained. The catalytic activity sedimented in the same position as in the control, accompanied by a peak of protein bound cAMP, indicating that a ternary complex holoenzyme-cAMP was formed. If cAMP was omitted from the gradient, catalytic activity showed the same profile, but the peak of protein bound cAMP was no longer observed; however, cAMP-binding activity could be detected by assay, cosedimenting with the catalytic activity (data not shown). These results seem to indicate that the cyclic nucleotide is weakly bound to the holoenzyme. Fig. 1C shows the sedimentation behaviour obtained when the protein kinase was completely dissociated with 2 μM [^{3}H]-cAMP and 0.5 M NaCl (Fig. 1C). The [^{3}H]cAMP associated to the regulatory subunit was not lost after exhaustive dialysis and chromatography through DEAE-cellulose column. These results indicate that cAMP is strongly bound to the protein.

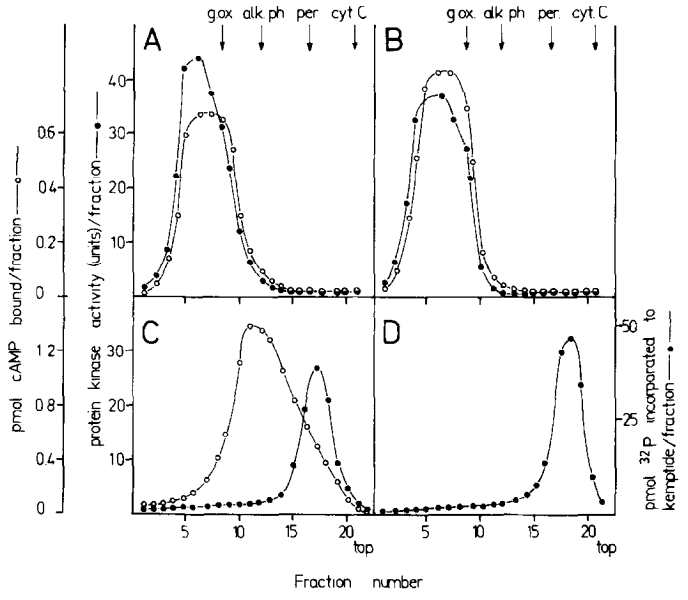

Fig. 1. Sucrose density gradient centrifugation of protein kinase. Preincubations and sucrose gradients were performed in buffer I, with the additions indicated in each case. In cases A, B and C, 250 units of protein kinase activity were preincubated at 30°C for 1 min in a final volume of 0.2 ml. Additions: (A) none; (B) 2 μM [^{3}H]cAMP in the preincubation mixture and 0.13 μM [^{3}H]cAMP in the gradient; (C) 5 μM [^{3}H]cAMP and 0.5 M NaCl in the preincubation mixture, 0.13 μM [^{3}H]cAMP and 0.5 M NaCl in the gradient. In case (D) 8 units of protein kinase activity were preincubated 1 min at 30°C in the complete incubation mixture of protein kinase. The gradient was prepared with the following additions: 0.75 mg/ml histone, 0.05 mM ATP, 10 mM $MgCl_2$ and 0.15 μM cAMP. This small amount of enzyme had to be used in order to prevent precipitation with histone. Aliquots were assayed for protein kinase activity using histone as substrate for gradients A-C and kemptide for gradient D (—•—). In gradients B and C aliquots from each fraction were filtered directly to measure the [^{3}H]cAMP bound (—o—). In gradient A aliquots were assayed for cAMP binding activity after 60 min incubation at 4°C with 140 nM [^{3}H]cAMP (—o—).

Under the conditions of the standard protein kinase assay, the holoenzyme was completely in the dissociated state as can be seen in Fig. 1D. At the enzyme concentration used in the experiment of the figure, cAMP alone produced less than 20% dissociation of the enzyme (data not shown). These results indicate that the ternary complex is not the active phosphorylating form of the enzyme.

Dissociation rate experiments

As can be seen in Fig. 2A, the amount of [^{3}H]cAMP bound to the holoenzyme decreased rapidly, with a half life of 2.5 min. This decrease is the

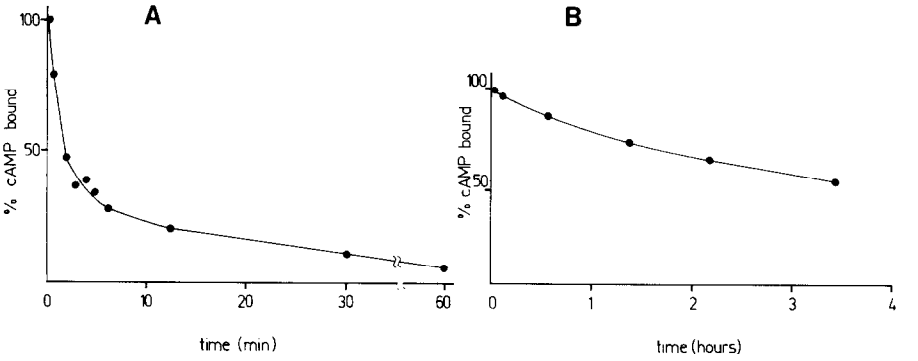

Fig. 2. Dissociation rate at 4°C of the holoenzyme and R subunit. (A) 500 units of protein kinase activity preincubated 60 min at 4°C with 60 nM [^{3}H]-cAMP were incubated in buffer I with 540 nM unlabeled cAMP in a final volume of 1.3 ml. (B) An aliquot of R subunit preparation (250,000 cpm of [^{3}H]cAMP bound), in a final volume of 1.3 ml was incubated in buffer I with 0.1 mM unlabeled cAMP. Aliquots of 75 µl were removed from each sample at the time points indicated and the amount of [^{3}H]cAMP bound determined by the membrane filtration method. 100% represents 14,000 cpm in both cases.

result of the exchange of bound [^{3}H]cAMP in the ternary complex with the unlabeled cAMP in the reaction mixture, since the amount of total cAMP used did not produce dissociation of the enzyme. The data given in Fig. 2B clearly show a slow isotopic exchange of [^{3}H]cAMP bound to the regulatory subunit (half life of approximately 210 min), quite different from the one depicted for the holoenzyme. The differences in the rates of isotopic exchange of holoenzyme and regulatory subunit are not unexpected, since the same qualitative results had been obtained from the experiments commented in the ultracentrifugation section.

Comparison of the amount of cAMP bound to holoenzyme and to R

Results of Table I show that maximum binding of [^{3}H]cAMP to protein kinase was attained at 140 nM cAMP. Under these conditions, the enzyme was in the undissociated state (Fig. 1B). When the incubation was performed under dissociating conditions (see Fig. 1C) the amount of cAMP bound was approximately doubled (line 5). These results indicate that the regulatory subunit has a greater binding capacity than the holoenzyme. Similar conclusions were obtained from the comparison of gradients 1A and 1C, since the total area of the binding capacity measured in gradient 1C was again approximately twice

the one of gradient 1A. The increase in the cAMP bound to protein under dissociating conditions (line 5) cannot be ascribed to the high salt concentration influence described by several authors (20-22), due to the fact that the samples were always diluted 20 times immediately before filtration.

cAMP-binding to holoenzyme and regulatory subunit analyzed by polyacrylamide gel electrophoresis

Protein kinase submitted to electrophoresis in a 5% polyacrylamide gel showed a single peak of catalytic and cAMP-binding activity migrating with a same R_f of 0.17 (Fig. 3A). A gel run in parallel, stained with Coomassie Blue showed a band migrating with the same R_f. This band completely disappeared if the enzyme was dissociated with NaCl and cAMP. Protein kinase preincubated with a saturating concentration of [^{3}H]cAMP (as in Fig. 1B) and submitted to electrophoresis showed the profile of Fig. 3B. Catalytic activity migrated in the same position as the control holoenzyme and a significative amount of [^{3}H]cAMP, estimated by direct scintillation counting of half slices, comigrated with the catalytic activity. These results indicate that a ternary complex has been formed between the cyclic nucleotide and the holoenzyme. Free [^{3}H]-cAMP migrated as a sharp peak accompanying the tracking dye. The presence of [^{3}H]cAMP in the complex, even in the abscence of [^{3}H]cAMP in the upper reservoir, raises the question of why did this bound cAMP resist the electrophoretic process and was lost after ultracentrifugation, but of course it must be born in mind that diffusion and dilution conditions are dramatically different under both experimental set-ups. The peak of [^{3}H]-label did not represent [^{3}H]cAMP bound to free regulatory subunit, since this complex submitted to electrophoresis migrated as a broad area with two principal peaks of R_f 0.46 and 0.60 (Fig. 3C). The broadness of the radioactive area was probably due to proteolytic attack of the regulatory moiety already reported to occur in different tissues (20, 23-25). Unfortunately we cannot give an R_f value for the catalytic subunit, since the efforts done to detect catalytic activity in the gel eluates, sensitizing the assay by using kemptide as

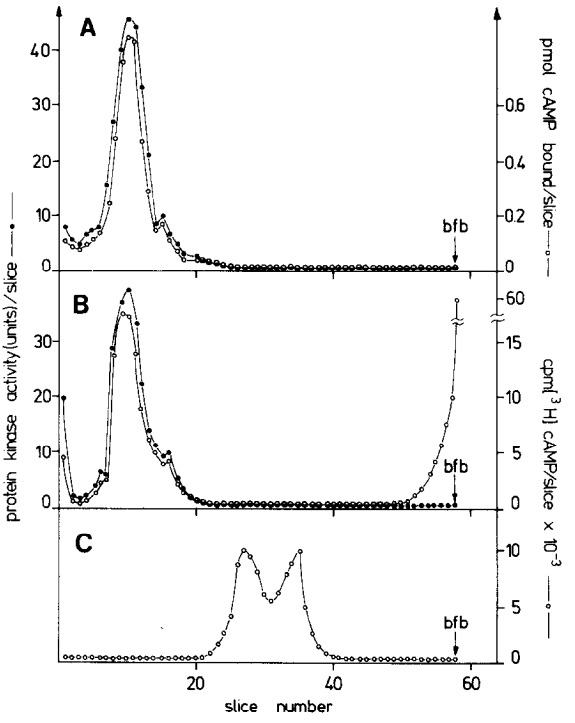

Fig. 3. Polyacrylamide gel electrophoresis of holoenzyme and R subunit. Gels A and B were loaded with 500 units of protein kinase preincubated 1 min at 30°C without (A) or with 150 nM [^{3}H]cAMP (B). Gel C was loaded with 200 µl of R - [^{3}H]cAMP subunit preparation (200,000 cpm). After completion of electrophoresis (approximately 4 h), gels were cut into 2 mm slices and the complete slice (A) or half ot it (B) were incubated with continuous shaking for 14 h at 4°C in 200 µl of 10 mM Tris-HCl buffer pH 7.4, containing 4 mM mercaptoethanol, 50% glycerol and 1 mg/ml bovine serum albumin. Protein kinase activity was assayed in an aliquot of the eluates, using histone as substrate (— • —). cAMP-binding activity of gel A was measured by incubating an aliquot of the eluates, 60 min at 4°C with 150 nM [^{3}H]cAMP (— o —). [^{3}H]cAMP bound to protein in gels B and C was estimated by dissolving the half slice (B) or the whole slice (C) in 0.3 ml of H_2O_2 30%, 30 min at 100°C and counting the radioactivity in a scintillation mixture containing Triton X-100: toluene (30:70).

substrate and very high specific activity for ATP, were unsuccesful. This negative result can be explained by the lability of the catalytic activity already reported (13). Another possibility is that the isoelectric point of the catalytic subunit of the Mucor enzyme was sufficiently high (as reported for the catalytic subunit of other sources (26)), so as to prevent the protein from entering the gel at the pH used.

Conclusions

The results obtained by gel electrophoresis and sucrose density centrifugation in the present study provide physical evidence for the existence of a ternary complex holoenzyme-cAMP. In addition, our kinetic results indicating that cAMP is capable of binding to two different entities, reinforce the idea that one of these entities is the holoenzyme, thus forming the ternary complex. In our system, at least, other factors such as increased ionic strength, or the presence of histone and/or ATP-Mg^{++} are necessary to release active catalytic subunit from the complex. The role of each of these factors is under study.

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