Yeast cyclic AMP-dependent protein kinase

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We have purified cyclic AMP-dependent protein kinase from the yeast *Saccharomyces cerevisiae*. The purified enzyme was inactive in the absence of cyclic AMP and displayed two protein bands on SDS gel electrophoresis. One was identified as the cAMP-binding protein by chromatography on cAMP-agarose. M_r of the latter was 50000 while the catalytic subunit had an M_r of 59000. The enzyme accepted yeast phosphorylase, glycogen synthase and fructose 1,6-bisphosphatase as substrates. No inhibition by the mammalian protein kinase inhibitor was observed.

Yeast *Cyclic AMP-dependent protein kinase* *Phosphorylase* *Glycogen synthase* *Fructose 1,6-bisphosphatase*

1. INTRODUCTION

Cyclic AMP-dependent protein kinases play an important role in mediating cAMP action in higher organisms. In unicellular eukaryotes, however, little is known. In yeast, cAMP-mediated changes in enzyme activity have been reported for trehalase [1] and fructose-bis-phosphatase [2]. The glycogen metabolizing enzymes, glycogen synthase and phosphorylase, are known to be regulated by phosphorylation and dephosphorylation [3,4] but information about the possible involvement of cAMP-dependent protein kinase in their phosphorylation is lacking.

In our group, a Mg^{2+}-dependent phosphatase was purified [5] which dephosphorylates and inactivates phosphorylase. The reverse reaction was found to be catalyzed by a cAMP-independent protein kinase (called phosphorylase kinase) which has been enriched 2800-fold and characterized [15]. In contrast to these findings, we observed in crude preparations a cAMP-dependent protein kinase which also activated yeast phosphorylase. In order to evaluate whether phosphorylase kinase was identical to the catalytic subunit of cAMP-dependent protein kinase, the latter was separated from cAMP-independent phosphorylase kinase activity and characterized.

The purified cAMP-dependent protein kinase accepted yeast phosphorylase and glycogen synthase [6]. No evidence was found for an identity of phosphorylase kinase and the catalytic subunit.

2. MATERIAL AND METHODS

2.1. *Purification of enzymes*

Yeast phosphorylase was purified by a method developed in our laboratory [7] and glycogen synthase as in [3]. For the purification of cAMP-dependent protein kinase, 2 kg of commercial baker's yeast (DHW Hefe, Hamm) were suspended in buffer A (20 mM K-phosphate (pH 6.2), 20 mM mercaptoethanol, 20 mM EDTA, 2 mM PMSF, 10 mM benzamidine) broken and processed as in [8]. The streptomycin sulfate supernatant was brought to 40% saturation with ammonium sulfate and the precipitated proteins collected by centrifugation. The pellets were dissolved in buffer A and exhaustively dialyzed against the same buffer overnight. The dialysate (49 mg/ml protein)

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was stirred for 1 h with 1 l CM-Sephadex which was previously equilibrated with buffer A and the resin washed on a Büchner funnel. The resuspended resin was poured into a column (30×6 cm) and protein eluted with 0.3 M NaCl in buffer A. The eluate was then passed over a 4 ml alpha$_2$-macroglobulin column and dialyzed against buffer B (50 mM Na-acetate (pH 5.8), 10 mM mercaptoethanol, 10 mM EDTA, 2 mM PMSF, 10 mM benzamidine, 5% glycerol). The dialysate (3 mg/ml protein) was applied to a CM-cellulose column (2.5×8 cm) which was equilibrated with buffer B and protein eluted with a 50–500 mM Na-acetate gradient (500 ml). Fractions were collected and assayed for cAMP-dependent and -independent protein kinase and cAMP-binding activities. The active fractions were pooled (1.4 mg/ml protein), dialyzed against buffer C (10 mM Tris, pH 6.8), 10 mM EDTA, 10 mM mercaptoethanol, 10 mM benzamidine, 5% glycerol) and applied to a Cibacron F 3 GA-Sepharose column (12×1.5 cm). The cAMP-dependent protein kinase was eluted by a linear 0–0.5 NaCl gradient (250 ml) while phosphorylase kinase eluted at much higher salt concentrations (1.5 M NaCl). The purified enzyme contains no cAMP-independent phosphorylase kinase activity. Total yield of protein was 35 mg. R-subunit was prepared by dissociating the holoenzyme in cAMP-agarose and freed from cAMP as in [8]. The unbound protein of the cAMP-agarose was used as C-subunit.

2.2. *Assays*

Activation of phosphorylase in the absence and presence of cAMP (1.25 μM final concentration) was measured in a reaction mixture that contained equal volumes of phosphorylase (10–20 μg) and protein kinase in 20 mM Mes (pH 6.2), 1 mM EDTA, 5% glycerol. The reaction was started by the addition of ATP-Mg (1/10 mM final concentration). After 3 min aliquots were removed and phosphorylase activity determined [4]. The phosphorylation of phosphorylase was measured in an assay mixture containing 60 μl phosphorylase (1.3 mg/ml), $MgCl_2$ (13 mM) in Mes (20 mM (pH 6.2), 1 mM EDTA, 5% glycerol), 10 μl kinase and 20 μl [γ-^{32}P] ATP [9] (1.3 mM, 300 cpm/pmol).

After 30 s, 1 min and 1.5 min, 20 μl were removed from the mixture and incorporated radioactivity was measured as in [10]. In the presence of cAMP, 1.62 μM cAMP was added to the substrate. Controls were run in the absence of phosphorylase to correct for autophosphorylation of cAMP-dependent protein kinase.

Phosphorylation of histone was measured as in [8] with 300 μg histone per test. Cyclic AMP-binding protein was assayed by the Millipore filter technique [8].

2.3. *Gel electrophoresis*

SDS gel electrophoresis on disc gels was performed as in [11] and on slab gels as in [12].

2.4. *Material*

DEAE- and CM-cellulose were products of Whatman (Springfield Mill). CM-Sephadex and Sephacryl S-300 were purchased from Pharmacia (Uppsala). Cibacron F 3 GA–Sepharose was prepared by a modification of the method in [13] using cyanogen bromide activated Sepharose 4B (Pharmacia). Protein kinase inhibitor, histone II S, cyclic nucleotides and cAMP-agarose were purchased from Sigma (Heidelberg). Alpha$_2$-macroglobulin was a product of Boehringer (Mannheim).

3. RESULTS AND DISCUSSION

3.1. *Identification of regulatory and catalytic subunit*

The purity of the cAMP-dependent protein kinase after Cibacron F3GA-chromatography varied when different batches of commercial yeast were used for preparation (fig.1) [1,2]. In most cases a second Cibacron F3GA-chromatography was sufficient for final purification. Otherwise a second CM-cellulose-chromatography and gel filtration on Sephacryl S-300 were included.

The purified enzyme was totally inactive in the absence of cAMP and required different concentrations of the cyclic nucleotide for maximum activation depending on protein concentration (fig.2).

In order to identify the regulatory subunit, the holoenzyme was dissociated on a cAMP-agarose column (1 ml). The total kinase activity in the presence of cAMP applied to the column was recovered in the flow-through of the colums which contained only cAMP-independent kinase activity. The cAMP-binding protein was eluted with 10 mM

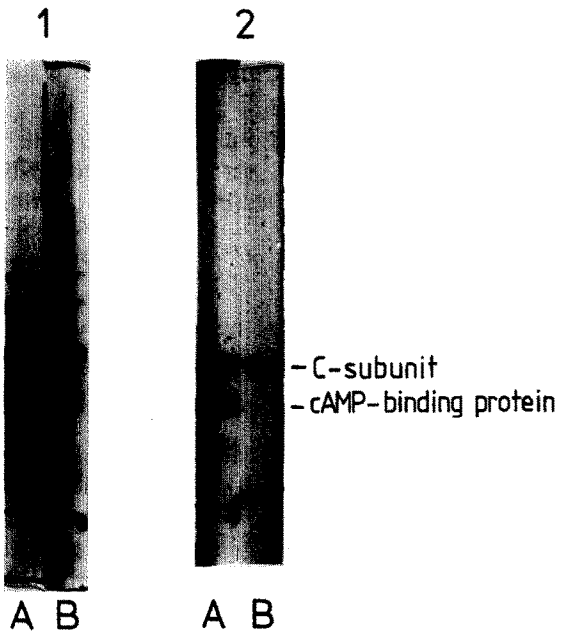

Fig.1. Identification of the R-subunit. Purified cAMP-dependent protein kinase (gel A), prepared from different yeast batches' gels [1,2], was subjected to SDS electrophoresis on 8% polyacrylamide disc gels as in [11]. Gel B shows the protein which was not bound to cAMP-agarose in the same preparation. About 5–20 μg of protein were applied.

cAMP as in [8], recovery of the protein, however, was poor.

As the unbound protein lacked one major protein band (fig.1B) this band referred to the cAMP-binding protein, the other to the catalytic subunit. The M_r of the catalytic subunit was 59 000, while that of the cAMP-binding protein was 50 000 (fig.3).

3.2. *Properties of the C-subunit*

All kinetic measurements were performed using the standard assays with phosphorylase as substrate. The pH-optimum was around 6.0 with 80% activity at 5.8 and 6.4. The apparent K_m in the presence of 10 mM $MgCl_2$ for ATP and GTP were 0.026 and 0.8 mM while CTP did not serve as phosphate donor. Optimal $MgCl_2$ concentration was 10–30 mM. The apparent K_m for histone was 1 mg/ml while concentrations above 5 mg/ml inhibited the enzyme.

Interaction of yeast kinases with mammalian R-subunit [8] and protein kinase inhibitor [14] has been reported and suggests a structural similarity between yeast and mammalian C-subunits. The catalytic subunit described here, however, was not inactivated by mammalian protein kinase inhibitor, had a much larger M_r than the C-subunits from higher organisms and phosphorylated serine as well as threonine. It has to be noted that an inhibitor-sensitive, cAMP-dependent protein kinase was observed in some preparations which differed from the purified enzyme with regard to

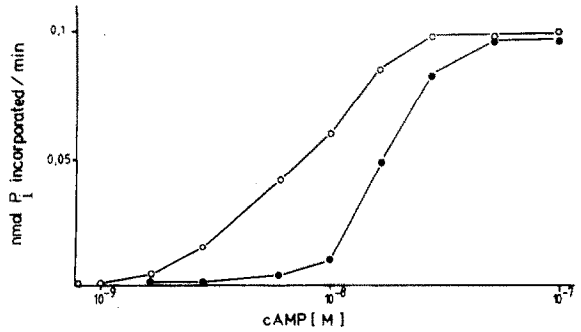

Fig.2. Activation of the purified kinase by cyclic AMP. Yeast phosphorylase was incubated with 10 nM (○—○) and 50 nM (●—●) cAMP-dependent protein kinase (final concentration) in the presence of different amounts of cyclic AMP, as indicated. Phosphorylation was determined in the standard phosphorylation assay.

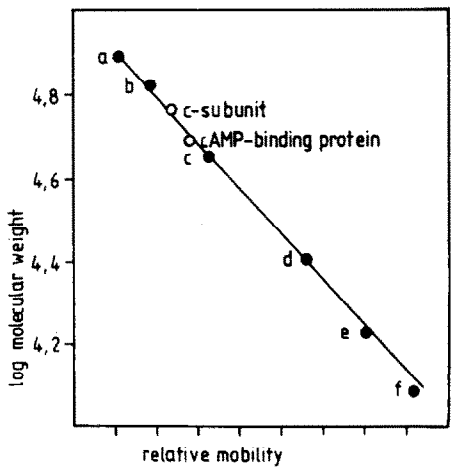

Fig.3. M_r-values of C- and R-subunit. cAMP-dependent protein kinase was applied to a 10% polyacrylamide slab gel calibrated with phosphorylase (M_r 100 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 43 000) and cytochrome *c* (M_r 12 500).

Table 1

Evidence for the non-identity of phosphorylase kinase and the catalytic subunit of cAMP-dependent protein kinase

	Phosphorylase kinase	Catalytic subunit
M_r	29 000	59 000
pH-optimum	8.0	6.0
Mg^{2+}-optimum	30 mM No inhibition at higher conc.	10–30 mM Inhibited at higher conc.
Substrates		
Phosphorylase[a]	0.5	0.15
Glycogen synthase[a]	0.2	0.8
Fructose-bis-phosphatase[b]	No phosphorylation	Phosphorylated
R-Subunit[a]	No phosphorylation	5

[a] Phosphorylation rates expressed as nmol $P_i \cdot min^{-1} \cdot mg\ protein^{-1}$ with 50 munits Histone kinase activity of phosphorylase kinase and cAMP-dependent protein kinase

[b] 100-Fold excess of phosphorylase kinase

the pH-optimum (~8.0) and Mg^{2+}-optimum (5–10 mM) but was not further characterized.

3.3. *Properties of the R-subunit*

The specificity of the R-subunit for binding of other cyclic nucleotides was tested by competitive studies in the cAMP-binding test, the assay contained 0.3 μM [^{3}H]cAMP and 10 mM subunit, the binding of [^{3}H]cAMP was inhibited 50% by concentrations of 0.7 μM, 40 μM, 0.7 mM and 0.3 mM of cIMP, cGMP, cCMP and cUMP, respectively. Incubation of the holoenzyme with ATP-Mg and cAMP led to autophosphorylation of the R-subunit. After acid hydrolysis of ^{32}P-labelled cAMP-binding protein phosphoserine and -threonine were identified (not shown).

Since the regulatory subunit of cAMP-dependent protein kinase described here shows the same properties regarding the M_r, autophosphorylation and binding specificity as the cAMP-binding protein purified in [8], it seems that they might be identical.

3.4. *Evidence for the non-identity of phospho rylase kinase and C-subunit*

Phosphorylase kinase and cAMP-dependent protein kinase are both able to phosphorylate yeast glycogen synthase and phosphorylase. Therefore, a possible identity of phosphorylase kinase with the catalytic subunit of cAMP-dependent protein kinase had to be considered. As shown in table 1, the kinases differ with regard to molecular mass, pH- and Mg^{2+}-optimum. In addition, neither phosphorylation of the regulatory subunit by phosphorylase kinase nor inhibition of the latter by the free cAMP-binding protein was observed [15]. Recently the phosphorylation of yeast fructose-bis-phosphatase by purified cAMP-dependent protein kinase was demonstrated [16]. Phosphorylase kinase however, showed no kinase activity in the same incubation mixture. The kinases are therefore most likely not identical.

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