Separation of a Cyclic 3′,5′-Adenosine Monophosphate Binding Protein from Yeast†

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ABSTRACT: A cyclic 3′,5′-adenosine monophosphate (cAMP) binding protein was isolated from several yeast strains. The protein interacted specifically with the 3′,5′-phosphodiester ring. At pH 7.4, the estimated binding constant of cAMP for the binding protein was 5 nM. This protein had a molecular weight of 24,000; it did not show any protein phosphokinase activity with or without cAMP, nor did it inhibit the protein phosphokinase isolated from yeast.

In recent work from this laboratory, a cyclic 3′,5′-adenosine monophosphate[1] binding protein was found in supernatant fractions of reticulocytes associated with protein phosphokinases (Tao *et al.*, 1970). A similar binding of cAMP to protein kinases was reported simultaneously by Gill and Garren (1970) in adrenal cortex extracts, and more recently in various tissues (Kurmon *et al.*, 1970; Reiman *et al.*, 1971). In these cases, the cAMP binds to and thereby dissociates an inhibitor protein from the catalytic portion of the complex.

In screening *Escherichia coli* and yeast supernatant fractions for cAMP binding activity, such activities were found but appeared not to be related to protein phosphokinase activity. Through the work of Zubay *et al.* (1970) and Emmer *et al.* (1970), an *E. coli* cAMP binding protein was associated with the release of catabolite inhibition. We want to report here on a cAMP binding protein which was found in yeast during fractionation of the cytoplasmic elongation factors (M. Salas and D. Richter, unpublished results) but could be separated from them; so far, no function can be assigned to it, and in particular, no relation to yeast phosphokinase (Rabinowitz and Lipmann, 1960).

Experimental Section

Growth Conditions. Saccharomyces fragilis was mostly studied and grown either in 15-l. flasks of a New Brunswick fermentor, or in 2-l. erlenmeyer flasks in a New Brunswick rotary shaking incubator. The yeast extract–peptone–salt medium was supplemented with 1.5 or 10% glucose, or with 2% sodium lactate (Richter, 1971). When the fermentor was used, a 10-l. culture was aerated (2 l. of air/min) and incubated at 37° with stirring (800 rpm). In experiments done in the shaking incubator, 500-ml cultures were incubated at 37° with shaking at 250 rpm. Growth was followed by measuring the absorbancy at 450 nm, and was stopped in the midlogarithmic phase. The culture was then rapidly cooled in an acetone–Dry Ice bath, and the cells were harvested in a continuous-flow rotor.

Isolation of the cAMP Binding Protein. Yeast cells (120 g wet wt) were suspended in two volumes of buffer (20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, and 1 mM dithiothreitol) and were disrupted in a French press cell at 15,000 psi. The homogenate was spun at 1000*g* for 10 min followed by a spin at 27,000*g* for 30 min. The S-100 fraction obtained by centrifugation at 100,000*g* for 2 hr was saturated with ammonium sulfate. The precipitated protein was reextracted three times successively with the following ammonium sulfate solutions (pH 6.8) that contained 1 mM dithiothreitol: 38.5, 28.7, and 21% (w/w). The 21% ammonium sulfate extract, which contained EF 2 and cAMP binding activity, was dialyzed against 5 mM phosphate buffer (pH 7.2) and 1 mM dithiothreitol. Of this extract, 1890 mg of protein was applied to a hydroxylapatite column (2.7 × 12 cm), preequilibrated with the same buffer. Fractions were eluted from the column in two steps: with 30 and 70 mM phosphate buffer (pH 7.2); both buffers contained 1 mM dithiothreitol. The 70 mM phosphate eluate contained EF 2 and cAMP binding activity, and was dialyzed against 20 mM Tris-HCl (pH 7.4)–1 mM dithiothreitol (buffer A) and further purified by DEAE-cellulose chromatography. To a DEAE-cellulose column (1.2 × 15 cm), which had been preequilibrated with 0.1 M KCl in buffer A, 61 mg of protein from the 70 mM phosphate step was applied. The cAMP binding protein was eluted with the same KCl concentration. This fractionation on DEAE-cellulose separated the cAMP binding activity from that of the cytoplasmic EF 2 activity, which was more retarded by the DEAE-cellulose (Richter and Lipmann, 1970). Further purification of the binding protein was achieved by a Sephadex G-150 chromatography. However, most of the experiments described here were performed with DEAE-cellulose fractions that were almost free of phosphodiesterase activity.

Assay for cAMP Binding Activity. Binding of [^{3}H]cAMP to protein was determined by the Millipore filter technique (Tao *et al.*, 1970). Binding protein (20–30 μg) and saturating amounts of cAMP were used (*cf.* Figure 5); 2.4×10^{-6} M [^{3}H]cAMP (6.35 Ci/mmole) was mixed in buffer B (20 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$) to a final volume of 50 μl and incubated for at least 15 min at 4° or for 3 min at 37°. A longer period of incubation at the elevated temperature required the presence of 0.5 mM dithiothreitol to maintain the activity. Incubation was terminated by dilution with 2 ml of ice-cold buffer B. The diluted reaction mixture was passed through a wet Millipore filter, which was then washed with 10 ml of cold buffer B and counted in 20 ml of Bray's scintillation fluid (Bray, 1960). At 4° an initial binding of cAMP

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[1] Abbreviations used are: cAMP, cyclic 3′,5′-adenosine monophosphate; cGMP, cyclic 3′,5′-guanosine monophosphate; cUMP, cyclic 3′,5′-uridine monophosphate; cCMP, cyclic 3′,5′-cytidine monophosphate; EF 2, elongation factor from eukaryotes, formerly called cytoplasmic G; PEI-cellulose, polyethyleneimine cellulose.

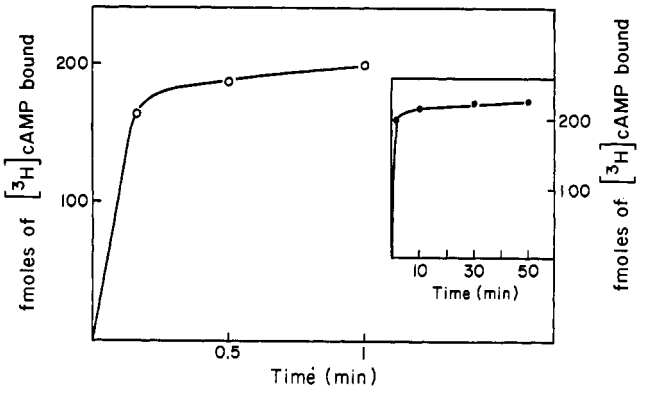

FIGURE 1: cAMP binding as a function of time. The incubation medium contained 15 mM potassium phosphate buffer (pH 7.6) and 20 μg of binding protein in a final volume of 65 μl. Incubations were at 4°, and the binding reaction was initiated by the addition of [^{3}H]-cAMP (16.3 Ci/mmole) to a final concentration of 1.4×10^{-6} M. Reactions were terminated, and protein-bound [^{3}H]cAMP was assayed as described in the Experimental Section.

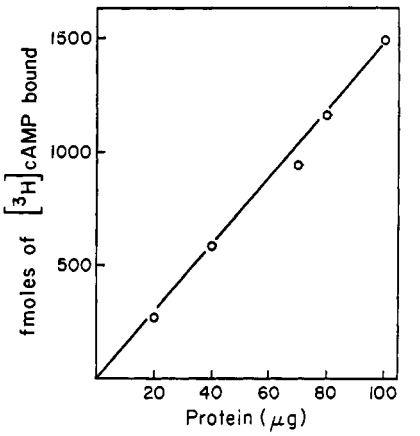

FIGURE 2: cAMP binding as a function of protein concentration. Conditions for cAMP binding assay were the same as in Figure 1, except that varying binding protein concentrations were used and incubation was at 4° for 60 min.

to about 80% saturation was very fast and slowed down considerably after 1 min, reaching a plateau at 60 min (Figure 1). It was proportional to the concentration of the binding protein (Figure 2). Mg^{2+} was not required, and pH 7–8 was optimal for cAMP binding.

Assay for Yeast Protein Kinase Activity. Kinase activity was measured in the presence of 20 mM Tris-HCl (pH 7.4), 100 mM KCl, 3 mM $MgCl_2$, 0.2 mM γ-[^{32}P]ATP (25 cpm/pmole), 200 μg of calf thymus histones or phosvitin, and protein kinase from yeast as indicated in the legends to the figures. As was shown by Rabinowitz and Lipmann (1960), the yeast protein kinase preferred phosvitin as substrate. Confirming these experiments, we found that when histones were used as substrate, less than 10% phosphorylation activity was obtained as compared to phosvitin. Therefore in the experiments with yeast kinase phosvitin was used as substrate. After incubation at 30° for 5 min, the reaction was stopped with 2 ml of 10% trichloroacetic acid. The mixture was passed through a glass fiber filter washed with 10 ml of 10% trichloroacetic acid, and counted in Brays' solution. The crude yeast protein kinase was obtained by ammonium sulfate fractionation (0 to 45%, pH 6.8) of the yeast S-100 fraction, and by chromatography on hydroxylapatite. Kinase activity was eluted from the column with 1.0 M phosphate buffer, pH 6.8, and dialyzed against 20 mM Tris-HCl buffer, pH 7.4, and 1 mM dithiothreitol. This preparation had no significant cAMP binding activity. Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Materials. The following yeast strains were used. *S. fragilis* (10022) and *Saccharomyces carlbergensis* (9080) came from American Type Culture Collection. The wild-type strain *Saccharomyces cerevisiae* 18A and the "petite" mutant II-1-40 (the latter lacking mitochondrial DNA), were obtained from Dr. J. Marmur. [^{3}H]cAMP and α-[^{32}P]ATP were obtained from Schwarz BioResearch, and PEI-cellulose thin-layer sheets from Brinkmann. γ-[^{32}P]ATP was purchased from ICN. Calf thymus histones and phosvitin were obtained from Sigma.

Results

Properties of the Yeast cAMP Binding Protein. Not unlike a crude reticulocyte EF 2 fraction (Tao *et al.*, 1970), the

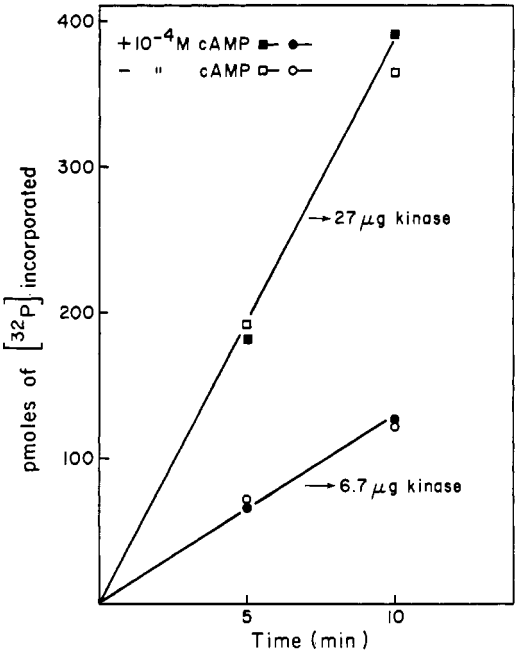

FIGURE 3: Absence of protein kinase stimulation by cAMP. The indicated amounts of enzyme were incubated as described in the Experimental Section using phosvitin as substrate, 0.2 mM γ-[^{32}P]-ATP (25 cpm/pmole) and if present, 10^{-4} M cAMP. Incubations were terminated by the addition of 2 ml of cold 10% trichloroacetic acid at the end of the time periods. Protein-bound ^{32}P was assayed as outlined in the Experimental Section.

counterpart from yeast cytoplasm also showed considerable cAMP binding activity; but extensive purification yielded a cAMP binding protein free of EF 2. All attempts to relate it to protein kinase activity have failed. As mentioned above, the isolation of a protein kinase from the yeast S-100 fraction has been previously reported (Rabinowitz and Lipmann, 1960) using phosvitin as a substrate. Preliminary experiments by Tao (unpublished data) had already indicated that this activity did not respond to cAMP. This was confirmed by experiments presented in Figure 3 showing the absence of any effect of cAMP on this enzyme. Since Tao *et al.* (1970) had found the activation of reticulocyte protein phosphokinase to be due to the release by binding of an inhibitor

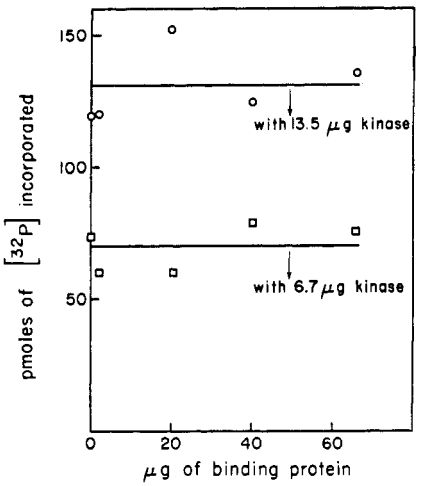

FIGURE 4: Absence of the inhibitory effect of the binding protein on yeast protein kinase. Yeast protein kinase (13.5 or 6.7 μg as indicated) was incubated in 100 μl of 20 mM Tris-HCl (pH 7.6), 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM dithiothreitol, 200 μg of phosvitin, 0.2 mM γ-[^{32}P]ATP (25 cpm/pmole), and varying amounts of cAMP binding protein. Incubations were at 30° for 5 min, and were terminated by the addition of 2 ml of 10% trichloroacetic acid. Protein-bound ^{32}P was assayed as outlined in the Experimental Section.

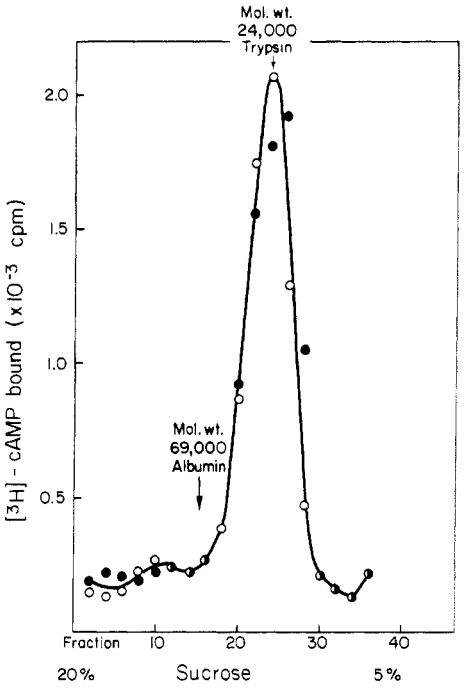

FIGURE 5: Analysis of the cAMP binding protein by sucrose density centrifugation. The purified binding protein (210 μg, DEAE-cellulose step) was layered on to a linear 5–20% sucrose gradient in buffer B, and centrifuged for 16 hr at 250,000g (●). Two-drop fractions were collected from the bottom of the centrifuge tubes and analyzed for cAMP binding activity (see Experimental Section). In one experiment, the gradient contained, in addition, 10^{-6} M [^{3}H]cAMP (○); these fractions were analyzed immediately for bound [^{3}H]cAMP by the Millipore filter technique (Tao *et al.*, 1970). Trypsin and albumin as markers were layered on separate gradients and determined by their absorption at 280 nm.

TABLE I: Comparison of the Cytoplasmic and Mitochondrial cAMP Binding Activities.[a]

S-100 Fraction	pmoles of [^{3}H]cAMP Bound/mg of Protein
S. cerevisiae	
Cytoplasm	11.5
Mitochondria	1.1
S. fragilis	
Cytoplasm	19.1
Mitochondria	2.4

[a] Lactate-grown yeast cells from the mid- or late-logarithmic phase were treated with glusulase and lysed osmotically; mitochondrial and cytoplasmic fractions were separated by centrifugation. Mitochondria were washed and homogenized by passing through a French press cell (Richter and Lipmann, 1970). Both mitochondrial and cytoplasmic fractions were centrifuged at 105,000g for 2 hr. cAMP binding activity was determined as described in the Experimental Section.

protein to cAMP, our cAMP binding protein was tested for inhibition of yeast protein kinase. Figure 4 demonstrates that even when rather high concentrations of the binding protein were used, kinase activity was not affected.

Figure 5 shows the determination of the molecular weight of the binding protein with sucrose density gradient centrifugation. No alteration in its molecular weight was observed when centrifugation was carried out in the presence of cAMP. The molecular weight of the binding protein was calculated to be 24,000, analyzed either by gradient centrifugation (Figure 5) or by Sephadex G-150 gel filtration (not shown). It may be noted that on sucrose centrifugation of the crude S-100 fraction, an additional peak of cAMP binding activity in the region of higher molecular weight was found; its binding to cAMP was low and was not further investigated.

To decide whether the binding protein might possibly be of mitochondrial origin, the cytoplasm and mitochondria were isolated and assayed separately for cAMP binding activity. Table I demonstrates that most of the cAMP binding protein was present in the cytoplasmic fraction with only minor activity in the mitochondrial fraction. Since its molecular weight, like that of the cytoplasmic fraction, is 24,000, it probably represents contamination by the cytoplasmic cAMP binding protein.

The cAMP binding protein could be isolated from various yeast strains, including *S. fragilis*, *S. carlbergensis*, *S. cerevisiae* 18A, and the mitochondrial DNA-depleted mutant II-1-40. Under various growth conditions, the activity of the binding protein was unchanged, except at high, *e.g.*, 10% glucose, concentrations, when slightly less active binding protein preparations were obtained. Although we have not yet been able to establish a function for the binding protein, all our data showed that it had a rather high affinity for the 3′,5′-phosphodiester derivative. As shown in Figure 6, an apparent binding constant of cAMP for the binding protein was calculated to be 5 nM. 2′,3′-cAMP (Table II) did not compete with the cAMP binding site. Since a 90-fold excess of cGMP inhibited the binding of cAMP by 60%, the specificity of the binding protein for the nucleotide base was less pronounced. Even at concentrations as high as 0.24 mM, ATP did not compete with 2.4×10^{-8} M cAMP for the binding

TABLE II: Specificity of cAMP Binding Protein.[a]

Competing Nucleotide Added	Concn of Competing Nucleotide (M)	Bound [^{3}H]cAMP (pmole)	Inhibn (%)
Experiment 1			
None		1.0	
2′,3′-cAMP	2×10^{-4}	1.0	0
3′,5′-cGMP	1.66×10^{-4}	0.37	63
3′,5′-cUMP	1.66×10^{-4}	0.78	22
3′,5′-cCMP	1.66×10^{-4}	0.95	5
Experiment 2			
None		0.24	
ATP	1.2×10^{-4}	0.25	0
ATP	2.4×10^{-4}	0.25	0
5′-AMP	1.3×10^{-4}	0.23	4
5′-AMP	2.6×10^{-4}	0.17	29

[a] [^{3}H]cAMP concentrations were 1.87×10^{-6} M (6.35 Ci/mmole) in expt 1, and 2.4×10^{-8} M (16.3 Ci/mmole) in expt 2. Binding protein for the two experiments was from different preparations. Each assay in expt 1 contained 14 μg of protein, and those of expt 2 contained 20 μg of protein.

protein. cAMP appeared not to be altered by the binding to this protein since it was recovered intact after heating the [^{3}H]cAMP protein complex to 80° for 3 min. The released radioactive material, chromatographed on PEI-cellulose thin-layer plates, was identified as [^{3}H]cAMP.

Discussion

The experiments reported here clearly demonstrate that yeast cells contain a protein that specifically binds cAMP and that this protein cannot be related to protein kinase function. The cAMP binding protein was isolated from various yeast strains including mitochondrial DNA-depleted mutants. The specific activity of the protein was not affected by various growth conditions, except for a slight decrease in yeast cells grown in 10% glucose. Although we could not establish any function for this protein other than to bind cAMP specifically, it might play a regulatory function in the catabolic machinery as is known for the catabolite gene activator in *E. coli* (Zubay *et al.*, 1970; Emmer *et al.*, 1970) As in *E. coli*, glucose is also known for its repressive effect on inducible enzymes in yeast (Magasanik, 1961; Holzer, 1967; Linnane and Haslam, 1970); however, only in *E. coli* is it evident that this catabolite repression is under a positive control system which is regulated by the level of cAMP. In view of the presence in yeast of cAMP and adenylyl cyclase (Sy and Richter, 1972), one may consider whether cAMP might have a function in the induction of enzyme synthesis in yeast. In the following paper, data are presented that indicate that both intracellular concentration of cAMP and cyclase activity depend on the growth conditions.

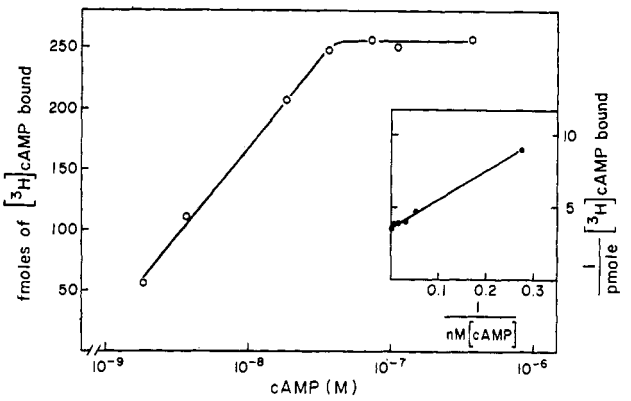

FIGURE 6: Binding of [^{3}H]cAMP as a function of cAMP concentration. Different amounts of [^{3}H]cAMP (16.3 Ci/mmole) were added to 50 μl of incubation mixture containing 20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, and 20 μg of binding protein. Incubations were performed at 4° for 60 min and terminated by dilution with 2 ml of ice-cold buffer B. Protein-bound cAMP was then assayed as described in the Experimental Section. The insert is a double-reciprocal plot for the estimation of binding constant.

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