CYCLIC AMP-DEPENDENT PROTEIN KINASE OF YEAST

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1. Introduction

Cyclic AMP-binding proteins with properties of a protein kinase regulatory subunit have been described in yeast with, however, conflicting reports on M_r-values. An M_r of 28 000 was reported in [1] and M_r 50 000 in [2]. Using photoaffinity labelling technique, a cAMP binding protein of 54 000 M_r was reported [3], while binding proteins of M_r 25 000–58 000 were reported in [4]. We have identified a low M_r (25 000) cAMP-binding protein from *Kluyveromyces (Saccharomyces) fragilis* [5]. The function of this protein was not known.

Cyclic AMP inhibits the outgrowth of *K. fragilis* from lag phase in minimal medium; the inhibition can be prevented by methionine or *S*-adenosyl methionine [6]. In searching for the underlying biochemical mechanism for this inhibition, we have initiated studies on cAMP-binding proteins of this organism. We found that *K. fragilis* contained 2 cAMP binding proteins: a 64 000 M_r protein which is the regulatory subunit of cAMP-dependent protein kinase and a 37 000 M_r protein which is derived from the regulatory subunit.

2. Materials and methods

Kluyveromyces fragilis (ATCC 10022) was maintained and grown as in [5]. [γ-^{32}P]ATP (2000 Ci/mmol) was obtained from Amersham, (Arlington Heights IL). Cyclic [^{3}H]AMP (32.3 Ci/mmol) was from New England Nuclear (Boston MA) and 8-azido-c[^{32}P]AMP (75 Ci/mmol) was a product of ICN (Irvine CA).

Abbreviations: cAMP, adenosine 5′,3′-monophosphate; 8-N_3-cAMP, 8-azido-adenosine 5′,3′-monophosphate; PMSF, phenyl methyl sulfonyl fluoride; PAB, *p*-amino benzamidine; M_r, relative molecular mass

2.1. *Preparation of cAMP-dependent protein kinase*

Frozen late-log phase cells were suspended in buffer A (20 mM Tris–acetate (pH 7.5), 10 mM KCl, 10 mM Mg-acetate, 2 mM dithiothreitol, and 0.1 mM EDTA) containing 0.5 mM PMSF and 10 mM PAB and were disrupted in a French press cell (15 000 lb. in^{-2}). After successive centrifugations at 15 000 × *g* for 30 min and 100 000 × *g* for 4 h, the supernatant (S100, 1.5 g protein) was applied to a DEAE-cellulose column (2.8 × 40 cm) pre-equilibrated with buffer A. Proteins were then eluted with a 10–500 mM KCl gradient. Fractions of 7 ml each were collected and assayed for cAMP-dependent and independent protein kinase and cAMP-binding activities. The active fractions (385 mg protein) were pooled and applied to a CM-cellulose column (2.8 × 35 cm) pre-equilibrated with 50 mM Na-acetate (pH 5.5), 10 mM Mg-acetate, 2 mM dithiothreitol, 0.1 mM EDTA and 0.5 mM PMSF. Proteins were then eluted with a 50–500 mM Na-acetate gradient. Fractions of 7 ml were collected and assayed for protein kinase and cAMP-binding activities. Cyclic AMP-dependent protein kinase was purified ~120-fold by the combined DEAE- and CM-cellulose column chromatography. A purification of ~1900-fold was obtained using an additional chromatofocusing column chromatography. However, most experiments were performed with the CM-cellulose purified enzyme.

2.2. *Protein kinase assay*

The enzyme sample was added to a reaction mixture (250 μl) containing: 40 mM Tris–acetate (pH 7.5), 5 mM Mg-acetate, 125 μg salmon sperm protamine, 1 mM dithiothreitol, 2 μM [γ-^{32}P]ATP (2 × 10^5 cpm), and if added, 1 μM cAMP. Samples were incubated

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for 3 min at 30°C and the reaction was terminated by the addition of 5 ml of cold 10% trichloroacetic acid. Samples were placed on ice for at least 15 min prior to being filtered on GF/C filters. The filters were washed, dried and radioactivity was counted in 5 ml of Hydrofluor.

2.3. *Cyclic AMP binding protein assay*

Cyclic AMP binding protein was assayed by the Millipore filter technique in [5].

2.4. *Photoaffinity label*

Cyclic AMP binding proteins were labeled using 8-N_3-c[^{32}P]AMP (75 Ci/mmol) at 6 × 10^{-8} M final conc. as in [3]. After irradiation, proteins were precipitated by 10% trichloroacetic acid and the precipitates were rinsed with 1 ml water prior to resuspension in loading buffer (60 mM Tris–HCl (pH 6.8), 1% SDS, and 20 mM dithiothreitol). Sucrose was added to the solution and the samples were boiled for 3 min. Polyacrylamide (5.7–16.5%)–SDS slab-gel electrophoreses were done according to [7]. The gels were then stained with Coomassie blue and destained with 7.5% acetic acid in 4% glycerol. Destained gels were dried and autoradiographed.

3. Results

Only one cAMP-dependent kinase eluting at 75 mM KCl on DEAE-cellulose was detected. Table 1 shows that the kinase phosphorylated protamine and histone 2b equally well but was inactive towards phosvitin and casein. There was a 20–30-fold stimulation by cAMP.

Table 1
Substrate specificity of cAMP-dependent protein kinase

Exp.	Substrate	[γ-^{32}P]ATP incorporated (cpm)	
		−cAMP	+cAMP
1	Protamine	446	10 780
	Histone H2b	306	9874
	Histone, mixed	100	2244
	Phosvitin	218	82
2	Protamine	480	10 944
	α-Casein	370	546
	γ-Globulin	130	318

Protein kinase assays were performed as in section 2: 125 μg indicated protein was used as substrate for 42 μg enzyme protein in 250 μl incubation mixture; cAMP was added to 1 μM final conc. Reactions were performed at 30°C for 3 min and were terminated by the addition of 10% trichloroacetic acid

M_r-Values of the protein kinase were determined in the presence and absence of cAMP by sucrose density gradient centrifugation (fig.1). In the absence of cAMP, protein kinase and cAMP binding activities had the same est. M_r 230 000. In the presence of

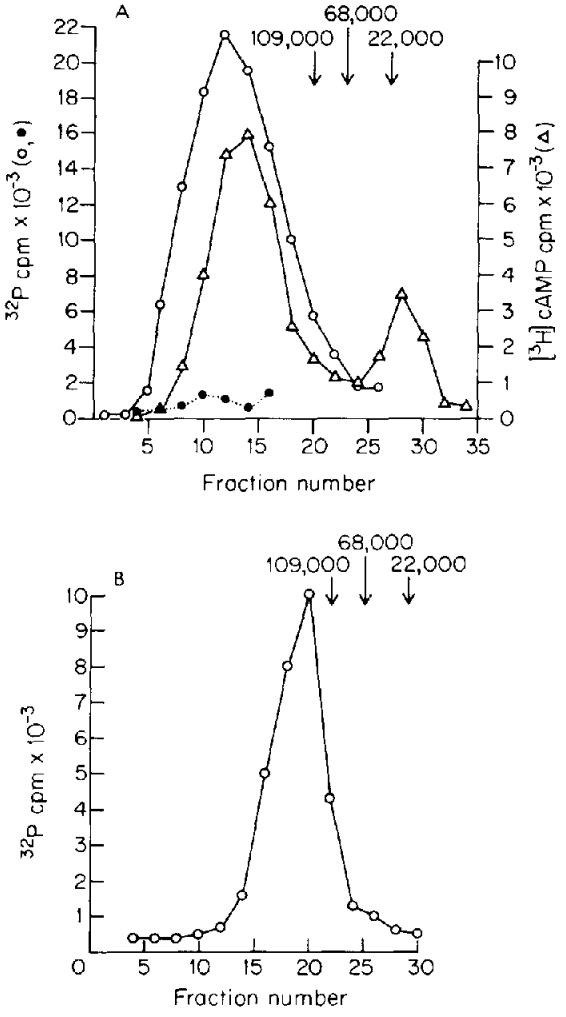

Fig.1. Sucrose density gradient centrifugation of cAMP-dependent protein kinase. The enzyme was centrifuged in a SW 41 rotor at 35 000 rev./min for 24 h on a 5–20% sucrose gradient containing 20 mM Tris–acetate (pH 7.5), 10 mM Mg-acetate, 10 mM KCl, 2 mM dithiothreitol, 0.1 mM EDTA; 350 μl fractions were collected and assayed as in section 2 for protein kinase activities in the presence (–○–) and absence (–●–) of cAMP: (A) gradient contained no cAMP; (B) gradient contained 2 mM cAMP; (–△–) c[^{3}H]AMP binding activity.

cAMP, protein kinase activity sedimented as a 130 000 M_r protein. Similar M_r was obtained using both low (10 mM) or high (100 mM) KCl concentration in the gradient. The M_r of the cAMP-binding regulatory subunit was determined by photoaffinity labelling with 8-azido-c[^{32}P]AMP followed by SDS–polyacrylamide gel electrophoresis. An M_r of 64 000 was found (fig.2(1), fig.3). A minor binding protein of 37 000 M_r was also detected (fig.2(1)). 8-Azido-c[^{32}P]AMP binding was inhibited by cAMP but not by ATP or adenosine (not shown).

The yeast cAMP-binding regulatory subunit was found to be highly sensitive to trypsin digestion and

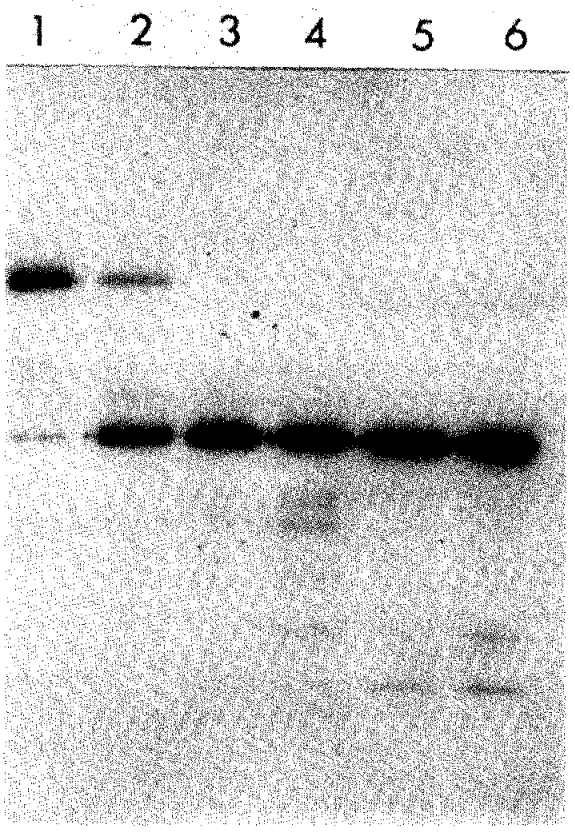

Fig.2. Cyclic AMP binding regulatory subunit and the effect of trypsin. Photoaffinity labelling of cAMP-dependent protein kinase was performed as in section 2. Protein (69 μg) was incubated at 4°C for 30 min with 6×10^{-8} M 8-N_3-c[^{32}P]-AMP and varying amounts of trypsin. The mixtures were then irradiated for 5 min with UV light and the reactions terminated by the addition of trichloroacetic acid. After electrophoresis, the gel was dried and autoradiographed. Lane (1) contained no trypsin; (2) 0.1 μg; (3) 0.5 μg; (4) 1 μg; (5) 5 μg; (6) 10 μg trypsin.

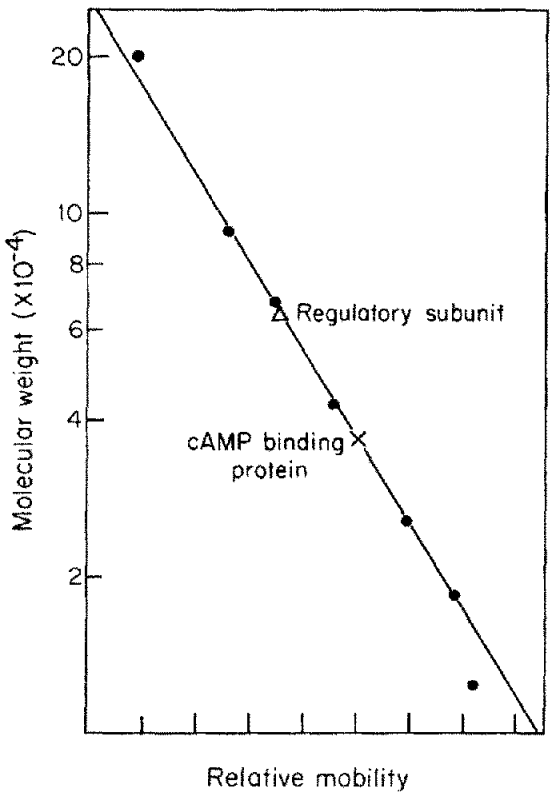

Fig.3. M_r-values of cAMP binding proteins. Experiments were performed as in fig.2. The following ^{14}C-labelled proteins (obtained from Bethesda Research Laboratory) were used as M_r standards: cytochrome *c*, 12 300; β-lactoglobulin, 18 400; α-chymotrypsinogen, 25 700; ovalbumin, 43 000; bovine serum albumin, 68 000; phosphorylase B, 92 500; and myosin (H-chain), 200 000.

that the product of a limited tryptic digest was a 37 000 M_r peptide that retained a cAMP binding site (fig.2,3). The 37 000 M_r binding protein was relatively stable toward trypsin even up to 100-fold higher trypsin concentration. We had isolated a cAMP-binding protein of 25 000 M_r as measured by sucrose gradient centrifugation [5]. This protein was found to be specific for cAMP but has no ascribable function to it. We have further purified this protein by cAMP–agarose affinity column chromatography [2] and found that it migrated in SDS–polyacrylamide gel electrophoresis as a 37 000 M_r peptide (fig.4 (6)) which is identical to the cAMP-binding trypsin-resistant core of the regulatory subunit (fig.4(3,4)).

The 64 000 M_r regulatory subunit and the 37 000 M_r protein were the only cAMP-binding proteins detected in S100 by photoaffinity labelling technique

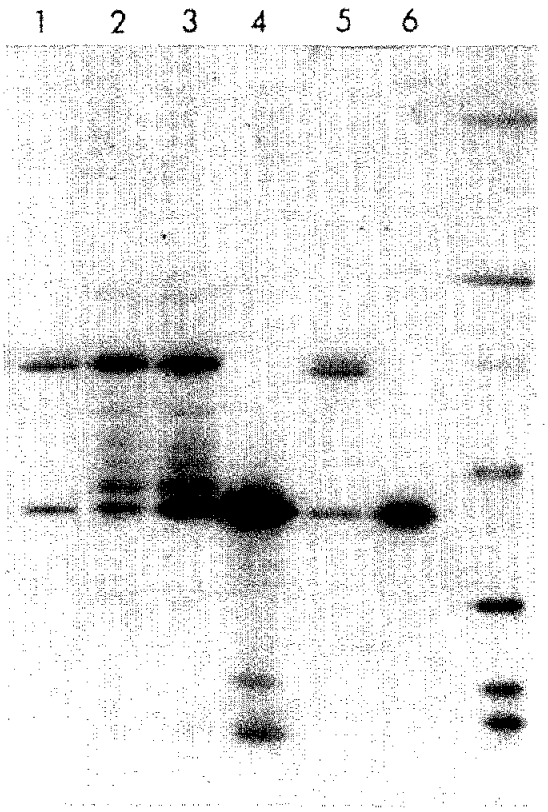

Fig.4. Cyclic AMP-binding proteins in yeast. Photoaffinity labelling with 8-N_3-c[^{32}P]AMP (6×10^{-7} M) were performed as in fig.1. Lane (1) contained S100 (256 μg protein) preparation and was incubated with 10^{-4} M AMP, 1.5 mM theophylline in addition to the standard reaction mixture. Lanes (2–4) contained 36 μg CM-cellulose purified cAMP-dependent protein kinase and were incubated with 0, 0.1 μg and 10 μg trypsin, respectively. Lane (5) contained 120 μg DEAE-cellulose purified cAMP-binding protein from S100. Lane (6) contained cAMP–agarose purified low M_r cAMP-binding protein. The last lane contained ^{14}C-labelled protein standards.

(fig.4(1)). A faint, slower migrating cAMP-binding band near the 64 000 M_r regulatory subunit band can be detected (fig.4(1,5)). The intensity of this band varied from preparation to preparation. Preliminary experiments indicate that the slower moving band is the phosphorylated form of the 64 000 M_r regulatory subunit. Mammalian type II regulatory subunits also migrate as a slower moving band when autophosphorylated [8,9].

4. Discussion

We have found that the M_r of the cAMP-dependent protein kinase from *K. fragilis* (230 000) differed significantly from that reported for baker's yeast (58 000) [1]. The M_r of the *K. fragilis* enzyme is similar to those of type II kinases reported in higher organisms [9]. The previously reported low M_r-values of cAMP-binding proteins from yeast may be due to proteolytic cleavages as suggested in [2,3]. We found that ammonium sulfate fractionation, even in the presence of PMSF and PAB, of S100 was unsuitable for the preparation of this enzyme because it led to a rapid loss of cAMP-dependent activity possibly due to proteolysis. The yeast's cAMP-binding regulatory subunit (M_r 64 000) is the largest protein kinase regulatory subunit so far reported. It is very similar to type II regulatory subunit in that it has a 37 000 M_r cAMP-binding, trypsin-resistant, core and that it can be phosphorylated.

Acknowledgements

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