SOLUBLE AND MEMBRANE-BOUND CYCLIC AMP DIESTERASE ACTIVITY WITH A LOW MICHAELIS CONSTANT IN BAKER'S YEAST

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1. Introduction

A soluble cAMP* diesterase from baker's yeast, with a molecular weight of about 65 000, has been purified 20 000-fold to apparent homogeneity by Fujimoto et al. [1] and 8000-fold in this laboratory [2]. It is unusual in having no requirement for free divalent metal ions, i.e. its activity is not changed by the presence of high concentrations of EDTA [1,2]. The enzyme is inactivated by 8-hydroxyquinoline and dipicolinic acid [1], and by *o*-phenanthroline (Londesborough, unpublished work); simple thiols are mixed inhibitors; and our preparation is reversibly inactivated by exposure to buffers below pH 6. These properties suggest that the enzyme may contain tightly bound metal, but this has not yet been established.

Most tissues and organisms examined contain cAMP diesterase activity with two or more Michaelis constants, at least one of which is less than 10 μM. However, this purified yeast diesterase has a single, high Michaelis constant. Fujimoto et al. [1] reported a K_m of 250 μM between 4 mM and 1 μM cAMP and that crude extracts of yeast spheroplasts exhibited a single K_m between 1 mM and 1 μM cAMP. Our purified preparation has a K_m of about 100 μM between 2 mM and 0.2 μM cAMP (Londesborough, unpublished work). Furthermore, it can be calculated that yeast contains about 0.3 nmole/min/g fresh weight of cAMP diesterase activity at 0.2 μM cAMP due to the purified enzyme, which is much less than the activity of adenyl cyclase [3]. This paper describes a higher molecular weight soluble cAMP diesterase, and a possibly distinct membrane-bound enzyme, with Michaelis constants of about 0.15 μM. Both require free divalent metal ions and are not inhibited by mercaptoethanol.

* The abbreviation used is cAMP, adenosine 3',5' cyclic monophosphate.

2. Experimental methods

Commercial baker's yeast from our Rajamäki factories was homogenised with a Gifford-Wood Minimill in 10 mM K phosphate pH 7.5–1 mM $MgCl_2$–0.3 mM EDTA (Buffer A) containing 0.6 M mannitol. Over 90% of the cells were broken. The homogenate, containing 10 g yeast in 70 ml, was centrifuged successively 10 min × 3000 *g*, 30 min × 12 000 *g*, and 60 min × 105 000 *g*. The first two precipitates were washed with Buffer A containing 0.6 M mannitol, and all precipitates were finally suspended in Buffer A. Samples of the homogenate and the 105 000 *g* supernatant were dialysed against Buffer A before assay.

Enzymes were assayed at 30°C, hexokinase and NADH-oxidase by standard methods [4,5]. The hydrolysis of cAMP was measured directly, or by a modification of the method of Brooker et al. [6]. Standard reaction mixtures contained 0.1 M Tris–Cl pH 8.1, 10 mM $MgCl_2$, about 10^5 cpm/ml [^{3}H](G)-cAMP (New England Nuclear), and 0.2 μM or 500 μM cAMP. Linear initial rates were estimated from three time points. Direct assays were stopped and the remaining radioactive cAMP isolated as previously described [2,3], except that, to permit counting of

the tritium label, cAMP was eluted from the silica gel by washing the excised tlc spots with 1 ml of water. After centrifugation, 0.8 ml of supernatant was counted in Bray's scintillant [7]. For the modified method of Brooker et al., 0.2 ml reaction mixtures were stopped by immersion in boiling water for 2 min, and then incubated with 0.2 ml of 1 mg/ml Crotalus adamanteus venom (Sigma) for 30 min at 30°C. 1.2 ml of 30% (w/v) Dowex 2–X8 (Cl^- form, Merck) was added, the mixture shaken for 15 min, and 0.5 ml of the supernatant counted in Bray's scintillant (cf. [8]). The effect of substrate concentration was investigated with the snake venom assay, which is more precise than the direct assay, although subject to systemic error in some tissues (cf. [9]). The concentration of unlabelled cAMP was varied in the presence of a constant amount (usually 0.0045 μM) radioactive cAMP, and the incubation time, rather than the enzyme concentration, was varied to obtain linear initial rates.

3. Results and discussion

3.1. *Distribution of activity amongst subcellular fractions*

About 85% of the total activity at 500 μM cAMP was found in the 105 000 *g* supernatant (table 1). Nevertheless, 13% precipitated between 12 000 *g* and 105 000 *g*, which is about twice the proportion of hexokinase in this fraction. These results confirm earlier reports [1,2]. However, when the activity was measured at 0.2 μM cAMP, 32% was found in the 105 000 *g* precipitate, and only 29% in the supernatant. This suggests that baker's yeast contains a membrane-bound cAMP diesterase with a low Michaelis constant, which makes only a small contribution (about 8%), to the total activity at 500 μM cAMP. The enzyme is not strongly bound to membranes, because if yeast is broken and fractionated in 0.3 M KCl, instead of 0.6 M mannitol, the distribution of diesterase activity at 0.2 μM cAMP resembles that of hexokinase (table 1).

Yeast contains between 0.04 and 0.6 μM cAMP depending on growth conditions [10,11]. It is notable that the total cAMP diesterase activity at 0.2 μM cAMP (pH 8.1, 10 mM $MgCl_2$) is close to the total adenyl cyclase activity (pH 6.8, 5 mM $MnCl_2$, 2 mM ATP) of 6 nmol/min/g yeast [3]. Both activities may be modulated in vivo, but at least their basal rates are roughly equivalent at physiological cAMP concentrations.

3.2. *Properties of the 105 000 g precipitate diesterase*

In contrast to the hightly purified soluble enzyme [1,2], the membrane-bound diesterase was slightly activated by mercaptoethanol, and almost completely inhibited by excess EDTA (table 2). It exhibited linear kinetics between 0.5 and 0.005 μM cAMP (data above 0.02 μM are shown in fig.1), with a K_m of about 0.12 μM. Above 0.5 μM the double-reciprocal plot has a small downward deviation, possibly due to contamination with the supernatant enzymes, but at 100 μM the

Table 1
Distribution of cAMP diesterase activity in subcellular fractions of baker's yeast

	Whole homogenate	1000–3000 *g* Precipitate	3000–12 000 *g* Precipitate	12 000–105 000 *g* Precipitate	105 000 *g* Supernatant
Protein (mg)	N.D.	N.D.	13	25	38
cAMP Diesterase (nmol/min)					
(a) at 500 μM cAMP	140	N.D.	10	18	120
(b) at 0.2 μM cAMP	8.5	0.1	0.9	2.7	2.5
(c) at 0.2 μM cAMP (KCl fractionation medium)	7.2	N.D.	N.D.	0.3	6.2
Hexokinase (μmol/min)	105	1.2	2.2	5.5	94
NADH-Oxidase (μmol/min)	30.2	2.5	23.1	2.4	0.2

Fractions were prepared and assayed by direct measurement of the disappearance of cAMP as described in the Experimental Section, except that in experiment (c) the yeast was broken and fractionated in Buffer A containing 0.3 M KCl instead of 0.6 M mannitol. Results are mean values per gram fresh yeast from at least two experiments. N.D., not determined.

Table 2
Effect of assay conditions on the diesterase activity in the 60 min × 105 000 *g* precipitate (P) and supernatant (S) fractions

Fraction	P	S	S
cAMP concentration (μM)	0.2	0.2	500
Assay conditions:			
Control	100	100	100
+10 mM Mercaptoethanol	115	95	40
No $MgCl_2$; +25 mM EDTA	1.3	12	108
+1.9 mM Theophylline	54	32	N.D.
+1.5 mg/ml NaDeoxycholate	180	57	N.D.

Activities were measured by the direct assay described in the Experimental Section, and are expressed as percentages of the respective control. N.D., not determined.

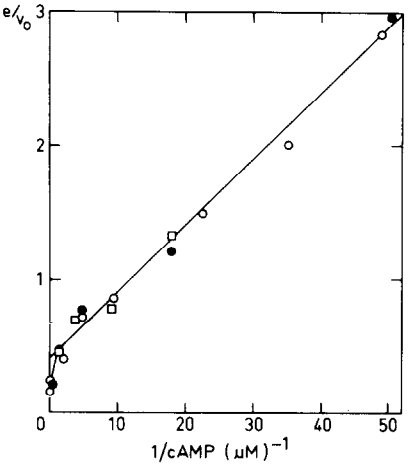

Fig.1. Kinetics of the cAMP diesterase activity in the 105 000 *g* precipitate. Data from 3 experiments, (□) (○) and (●) respectively, are shown, covering cAMP concentrations between 0.02 μM and 109 μM. e/v_0 is in arbitrary units.

Table 3
Reversible dissociation of cAMP diesterase activity from 105 000 *g* precipitate by 0.3 MKCl

	Total activity (nmol/min)		Percent recovery
	Precipitate	Supernatent	
(1) Original 105 000 *g* precipitate and supernatant from 10 g yeast	19.8	10.2	–
(2) Precipitate from (1) washed by suspension in 36 ml Buffer A containing 0.6 M mannitol	17.8	2.0	(100)
(3) Precipitate from (2) suspended in 36 ml Buffer A containing 0.3 M KCl	1.59	12.5	79
(4) 50% of precipitate from (3) mixed with 41% of supernatant from (3) and dialysed against Buffer A alone	5.75	0.70	103
(5) 50% of precipitate from (3) mixed with original 105 000 *g* supernatant from 2.5 g yeast and dialysed against Buffer A alone	3.28	1.19	133
(6) 6.5 ml of supernatant from (3) dialysed by itself against Buffer A alone	0.38	1.95	(100)

The 105 000 *g* precipitate obtained as described in the Experimental Section was treated as shown on the left. After each stage the material was again centrifuged for 60 min × 105 000 *g*. Samples of the precipitate were suspended in Buffer A, and samples of the supernatants dialysed against Buffer A before assay at 0.2 μM cAMP by the modified procedure of Brooker et al. described in the Experimental Section.

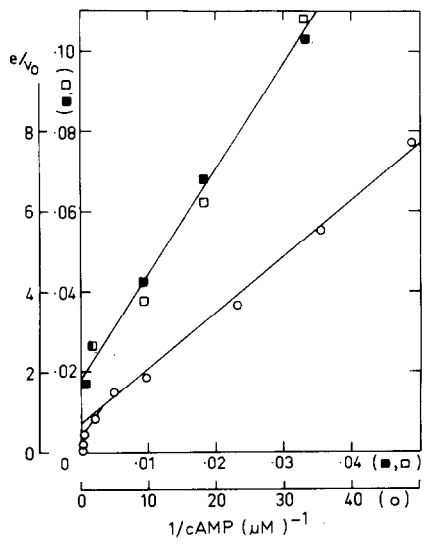

Fig.2. Kinetics of the cAMP diesterase activity in the 105 000 *g* supernatant. Data between 0.02 μM and 109 μM cAMP (○) were obtained with a final enzyme concentration of 73 μg/ml, and data between 30.3 μM and 1800 μM cAMP with enzyme concentrations of 73 μg/ml (□) and 730 μg/ml (■).

velocity is still less than 4 times the V_{max} obtained from the linear part of the plot.

The activation by Na deoxycholate was accompanied by a partial solubilisation of the enzyme (Tikanoja and Londesborough, unpublished work). About 85% of the activity was also solubilised by incubation with 0.3 M KCl, and the activity so released became membrane-bound again when the KCl was removed (table 3). Incubation in Buffer A of KCl-treated 105 000 *g* precipitate with the original (mannitol) 105 000 *g* supernatant resulted in the binding to the membranes of a large fraction of the supernatant diesterase activity at 0.2 μM cAMP. This raises the possibility that the same protein is reponsible for this activity in both the 105 000 *g* precipitate and supernatant. Factors affecting the binding are currently being examined to find out if the activity is membrane-bound under physiological conditions.

3.3. *Properties of the 105 000 g supernatant diesterase*

The supernatant exhibited marked biphasic kinetics (fig.2). The double-reciprocal plot has two linear regions with Michaelis constants of about 0.2 μM and 150 μM and V_{max} values differing by a factor of 40.

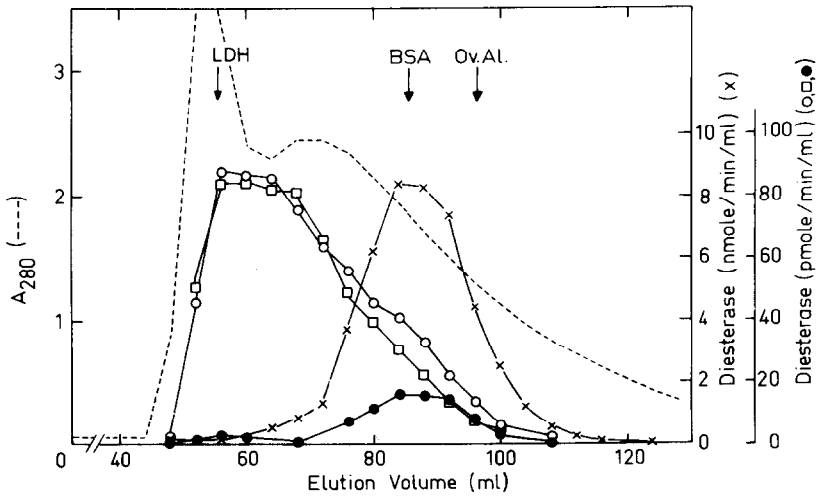

Fig.3. Gel-filtration of the 105 000 *g* supernatant on Sephadex G150. The supernatant was concentrated with $(NH_4)_2SO_4$, (50 g%), and 4.0 ml applied to a 2.9 × 30 cm column equilibrated with Buffer A. Diesterase activity was determined by the modified procedure of Brooker et al., described in the Experimental Section, under the following conditions: 500 μM cAMP (×); 0.2 μM cAMP (○); 0.2 μM cAMP + 24 mM mercaptoethanol (□); 0.2 μM cAMP, no $MgCl_2$ + 25 mM EDTA (●). Arrows mark the approximate exclusion volumes of rabbit muscle lactic dehydrogenase (M.W. 135 000), bovine serum albumin (M.W. 68 000), and ovalbumin (M.W. 45 000).

Evidence that this kinetic behaviour is due to the existence of more than one cAMP diesterase in the 105 000 *g* supernatant is presented in table 2. At 500 μM cAMP, the diesterase activity of the unpurified supernatant resembles the highly purified, 65 000 mol.wt., soluble enzyme in being strongly inhibited by mercaptoethanol, but insensitive to the removal of divalent metal ions. However, at 0.2 μM cAMP the activity is nearly insensitive to mercaptoethanol, but is 88% inhibited by excess EDTA. The residual activity at 0.2 μM cAMP in the presence of 25 mM EDTA (about 0.3 nmole/min/g yeast), is about that expected to be shown at this cAMP concentration by the 65 000 mol.wt. enzyme, which has an activity of 120 nmole/min/g yeast at 500 μM cAMP in the supernatant (table 1) and a K_m of about 100 μM [1,2].

3.4. *Separation of high and low K_m diesterase activity by gel-filtration of the 105 000 g supernatant*

Gel-filtration of the 105 000 *g* supernatant on Sephadex G150 (fig.3) revealed a roughly symmetrical peak of activity at 500 μM cAMP with about the same elution volume as bovine serum albumin, as previously reported [1,2]. This peak was coincident with a peak of activity at 0.2 μM cAMP *not* inhibited by 25 mM EDTA, and the ratio of these two activities at their peak (8.3 nmol: 16.6 pmol/min/ml, fig.3) is that expected for the 65 000 mol.wt. high K_m enzyme, (see above). The main peak of diesterase activity at 0.2 μM cAMP was at a much smaller elution volume and was almost completely suppressed by EDTA, but not affected by 24 mM mercaptoethanol. This peak was very broad. Furthermore, even at the elution volume of ovalbumin, about half the activity at 0.2 μM cAMP was inhibited by EDTA and not inhibited by 24 mM mercaptoethanol, and therefore cannot be due to the 65 000 mol.wt. high K_m enzyme. These observations suggest that different protein molecules with molecular weights ranging approximately from that of BSA (68 000) to that of rabbit LDH (135 000) are reponsible for the EDTA-sensitive cAMP diesterase activity at 0.2 μM cAMP. It is not yet clear whether these molecules are related by association equilibria. However, the specific velocity of the 105 000 *g* supernatant at 0.1 μM cAMP appears to decrease with both the enzyme concentration in the assay mixture and the stock enzyme concentration (Londesborough, unpublished work). This behaviour could be explained by dissociation of the enzyme into less active subunits, although other possible explanations have not yet been excluded. At high cAMP concentrations, the specific velocity of the 105 000 *g* supernatant was independent of the enzyme concentration over the range examined (fig.2).

The experiments described show that yeast contains cAMP diesterase(s) with very low Michaelis constants, and activities at physiological cAMP concentrations comparable with that of adenyl cyclase. Under normal conditions, the concentration of cAMP in yeast is presumably determined by these enzymes and adenyl cyclase. The significance of the high K_m, 65 000 mol.wt. diesterase, however, remains obscure.

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