Characterization of an Adenosine 3′:5′-Cyclic Monophosphate Phosphodiesterase from Baker's Yeast

ITS BINDING TO SUBCELLULAR PARTICLES, CATALYTIC PROPERTIES AND GEL-FILTRATION BEHAVIOUR

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1. The 3′:5′-cyclic AMP phosphodiesterase in the microsomal fraction of baker's yeast is highly specific for cyclic AMP, and not inhibited by cyclic GMP, cyclic IMP or cyclic UMP. Catalytic activity is abolished by 30 μM-EDTA. At 30°C and pH 8.1, the K_m is 0.17 μM, and theophylline is a simple competitive inhibitor with K_i 0.7 mM. The pH optimum is about 7.8 at 0.25 μM-cyclic AMP, so that over the physiological range of pH in yeast the activity changes in the opposite direction to that of adenylate cyclase [pH optimum about 6.2; Londesborough & Nurminen (1972) *Acta Chem. Scand.* **26**, 3396–3398]. 2. At pH 7.2, dissociation of the enzyme from dilute microsomal suspensions increased with ionic strength and was almost complete at 0.3 M-KCl. MgCl$_2$ caused more dissociation than did KCl or NaCl at the same ionic strength, but at low KCl concentrations binding required small amounts of free bivalent metal ions. In 0.1 M-KCl the binding decreased between pH 4.7 and 9.3. At pH 7.2 the binding was independent of temperature between 5 and 20°C. These observations suggest that the binding is electrostatic rather than hydrophobic. 3. The proportion of bound activity increased with the concentration of the microsomal fraction, and at 22 mg of protein/ml and pH 7.2 was 70% at I 0.18, and 35% at I 0.26. Presumably a substantial amount of the enzyme is particle-bound *in vivo*. 4. At 5°C in 10 mM-potassium phosphate, pH 7.2, the apparent molecular weight of KCl-solubilized enzyme decreased with enzyme concentration from about 200000 to 40000. In the presence of 0.5 M-KCl, a constant mol.wt. of about 55000 was observed over a 20-fold range of enzyme concentrations.

Baker's yeast contains a soluble 3′:5′-cyclic nucleotide phosphodiesterase (EC 3.1.4.17) with a high K_m (about 100 μM) and a mol.wt. of about 65000 (Fujimoto *et al.*, 1974; Londesborough, 1974). In addition, yeast homogenates prepared in low-ionic-strength buffers contain both particle-bound and soluble cyclic AMP phosphodiesterase activities with K_m values close to 0.15 μM (Londesborough, 1975). The latter enzymes are probably mainly responsible for the destruction of cyclic AMP at the very low concentrations encountered under normal conditions. The particle-bound enzyme, which is called 'cyclic AMP phosphodiesterase I' in the present paper, dissociates from the particles at high salt concentrations, and may be identical with the soluble low-K_m enzyme. In recent years, several enzymes of the glycolytic pathway, usually regarded as cytosolic enzymes, have also been found to bind to plasma and intracellular membranes at low ionic strength, but to be dissociated at physiological salt concentrations (see, for example, Clarke & Masters, 1972; Kant &

Steck, 1973; Melnick & Hultin, 1970). The question arises as to whether such enzymes are particle-bound *in vivo*. In the present paper a study of several factors affecting the binding of cyclic AMP phosphodiesterase I is described, and it is concluded that a large fraction of the enzyme is particle-bound within the yeast cell. The catalytic properties of the enzyme were also examined, partly in the hope of finding behaviour which might suggest how the concentration of cyclic AMP is regulated in yeast. A preliminary account of some of this work has already been presented (Londesborough, 1976*b*).

Materials and Methods

Cyclic [G-^{3}H]AMP was obtained from NEN Chemicals G.m.b.H. (Dreieichenhain, West Germany). Unlabelled nucleotides and pig heart lactate dehydrogenase were from Boehringer G.m.b.H. (Mannheim, West Germany). *Crotalus atrox* (snake) venom, ox γ-globulins, ox serum albumin, ovalbumin,

horse muscle myoglobin and horse heart cytochrome c were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dowex 2 (X8) anion-exchange resin was purchased from Fluka A.G. (Buchs, Switzerland) and Sephadex G-100 and Dextran Blue 2000 were from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

Enzyme assay

Cyclic AMP phosphodiesterase activity was determined at 30°C in 0.1 M-Tris/HCl, pH 8.1, containing 5 mM-$MgCl_2$ and 0.25 μM-cyclic [G-^{3}H]AMP (about 250 c.p.m./pmol) by the previously described modification (Londesborough, 1976a) of the method of Brooker *et al.* (1968), except that 2.5 mg of albumin/ml was included in the reaction mixture and enzyme samples were diluted, if necessary, in 10 mM-potassium phosphate/1 mM-$MgCl_2$/0.1 mM-EDTA, pH 7.2 (Buffer A), containing 10 mg of albumin/ml. A milliunit of enzyme was defined as the amount which catalyses the destruction of 1 nmol of cyclic AMP/min under these conditions. Linear initial rates were estimated from zero (in practice about 5 s) and three or four time points spread over 3–15 min, as appropriate. When the total concentration of cyclic AMP was varied, the amount of cyclic [^{3}H]AMP (about 9 nM) was kept constant and was included when calculating the lower concentrations of cyclic AMP. When the pH was varied, the *Crotalus atrox* venom used in the second stage of the assay was suspended in 0.2 M-Tris/HCl, pH 8.1. Controls at the pH limits (pH 5.2 and 9.5) showed that the gain of radioactivity by the Dowex supernatants was equal to the loss from cyclic AMP, determined after isolation by t.l.c. as previously described (Londesborough, 1975).

Protein determinations

Protein was determined by the biuret reaction (Gornall *et al.*, 1949) with samples preincubated for 30 min in 5% (w/v) sodium deoxycholate. It was assumed that 1 mg of protein in 3 ml of final assay mixture caused an A_{540}^{1cm} of 0.1.

Preparation of the 105 000g sediment

Commercial baker's yeast (*Saccharomyces cerevisiae*) from our Rajamäki factories was broken and fractionated in Buffer A containing 0.6 M-mannitol as previously described (Londesborough, 1975), except that the constitution of Buffer A was slightly altered (to 10 mM-potassium phosphate/1 mM-$MgCl_2$/0.1 mM-EDTA, pH 7.2). The sediment collected between 12 000g and 105 000g was suspended in the modified Buffer A and usually stored for a few days at −20°C. No change in its enzymic activity was observed over several months' storage. Thawed material from 20 g pressed weight of yeast was diluted with Buffer A to 60 ml and again centrifuged for 1 h at

105 000g. The Buffer A-washed pellet obtained is referred to below simply as the '105 000g sediment', except in Table 1. It was suspended in Buffer A for studies of its catalytic activity, and in the appropriate buffers described below for studies of the dissociation of cyclic AMP phosphodiesterase I.

Dissociation of enzyme from the 105 000g sediment

Portions of the 105 000g sediment not yet washed with Buffer A were suspended in about 3 ml of Buffer A/material from 1 g of pressed yeast (except that in the experiment at pH 6.0 in Fig. 4 10 mM-potassium phosphate/1 mM-$MgCl_2$/0.1 mM-EDTA, pH 5.7, was used instead of Buffer A so that the pH of the suspension was 6.0) and centrifuged for 1 h at 105 000g. The pellet was dispersed with a glass rod, and suspended in 6–7 ml of the appropriate buffer to a known final volume. The pH of the suspension was measured with a glass electrode at 5°C. After 20–30 min equilibration, usually on ice, the suspension was centrifuged for 1 h at 105 000g. The volume, pH and cyclic AMP phosphodiesterase activity of the supernatant were measured at once. The sediment was suspended in Buffer A, and assayed, usually after storage at −20°C. The bound activity was calculated from the total sedimented activity by correcting for the amount of supernatant trapped in the sediment. The volume of particulate matter (in μl) was assumed to be twice the amount of

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Table 1. *Subcellular distribution of low-K_m cyclic AMP phosphodiesterase activity, and elution and concentration of the microsomal enzyme*

Yeast was disintegrated and fractionated in Buffer A/0.6 M-mannitol, and the cyclic AMP phosphodiesterase activity in the 105 000g sediment eluted and concentrated as described in the Materials and Methods section.

	Total activity (munits/g of pressed yeast)	Specific activity (munits/g of protein)	Recovery of microsomal activity (%)
Distribution			
Whole homogenate	11.5	0.12	—
12 000g sediment	0.7	0.04	—
105 000g sediment	5.4	0.17	(100)
105 000g supernatant	3.9	0.08	—
Elution and concentration			
Buffer A-washed 105 000g sediment	5.0	0.19	92
Buffer A/0.5 M-KCl extract	4.0	0.86	74
$(NH_4)_2SO_4$ precipitate:			
before dialysis	3.6	1.0	66
after dialysis	2.7	1.2	49

sedimented protein (in mg), and the difference between this volume and the observed volume of the sediment (i.e. the suspension volume minus the volume of recovered supernatant) was assumed to be trapped supernatant.

Preparation of concentrated KCl-solubilized enzyme

The 105000g sediment from 20g of yeast, washed with Buffer A as described above, was suspended in Buffer A/0.5 M-KCl to 60 ml. After 30 min equilibration on ice, the suspension was centrifuged for 1 h at 105000g. The supernatant was adjusted to 1 mM-EDTA by addition of 0.1 M-EDTA, pH 7.0, and then 4 g of $(NH_4)_2SO_4$ per 10 ml was added. After 15 min, the resulting precipitate was collected by centrifugation at 34000g for 30 min. It was dissolved in 2 ml of Buffer A and dialysed against two changes of 400 ml of Buffer A for a total of 6 h. A large precipitate formed in the dialysis sac. The clear supernatant obtained by centrifugation at 34000g for 10 min was used for gel-filtration and catalytic studies. Results of a typical preparation are shown in Table 1.

Gel filtration

Concentrated enzyme obtained as described above was used directly or after dialysis against Buffer A/0.5 M-KCl. Samples (1.5–4 ml) were applied at 5°C to columns (2.5 cm × ~35 cm) of Sephadex G-100 equilibrated with Buffer A or Buffer A/0.5 M-KCl. The columns were calibrated with Blue Dextran 2000 and standard proteins by the procedure of Andrews (1965), the apparent molecular weights observed by Andrews (1965) being used where these differed from the actual values. Fractions (about 3.5 ml) were collected with a drop counter. Elution volumes were measured from the mid-point of the applied sample and corrected for the dead space of connecting tubes. Cyclic AMP phosphodiesterase activity was measured as soon as the fractions became available.

Results

Effect of protein concentration on the specific activity

The specific activity of cyclic AMP phosphodiesterase I measured in the absence of albumin declined markedly with the final concentration of enzyme in the reaction mixture (Fig. 1). The dependence of the specific activity on enzyme concentration was similar with samples of 105000g sediment, or of enzyme solubilized from this sediment by washing with 0.2 M- or 0.5 M-KCl in Buffer A, or of solubilized enzyme precipitated with $(NH_4)_2SO_4$ and dialysed against Buffer A. Because the dependence of the specific activity on enzyme concentration was reproducible (except at final enzyme concentrations

less than about 10 μunits/ml, when the specific activity also depended on the stock-enzyme concentration), and because the apparent molecular weight of the enzyme also depends on the enzyme concentration (see below), it was possible that the variation of specific activity with enzyme concentration was caused by dissociation of the enzyme into a less active form. This does not appear to be the case, because, as shown in Fig. 1, the specific activity was almost constant over a sevenfold range of enzyme concentrations if enzyme samples were diluted in 10 mg of albumin/ml of Buffer A and assayed in reaction mixtures containing 2.5 mg of albumin/ml.

Instead, the decrease in specific activity with enzyme concentration is probably due to adsorption of the enzyme by the glass tubes in which samples were diluted and assayed. Thus at 0.1 mg of sample protein/ml of Buffer A, KCl-solubilized enzyme retained 47% of its activity after incubation for 40 min at 0°C in one glass tube, but only 26% after sequential incubations for 10 min in each of three glass tubes, and none when the solution was gently shaken for 30 min with an equal volume of glass beads (0.2 mm diam.) Further, the same enzyme sample retained 61% of its activity when incubated for 40 min at 0°C in a Nalgene (Du Pont Co., Newtown, CT, U.S.A.) tube. Attempts to recover
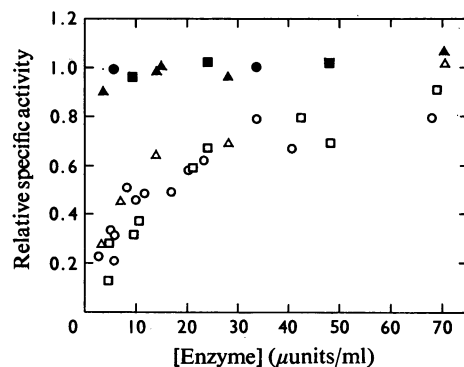

Fig. 1. *Effect of enzyme concentration on the specific activity*
The specific activities of enzyme diluted in Buffer A containing 10 mg of albumin/ml and assayed in reaction mixtures containing 2.5 mg of albumin/ml (●, ■, ▲) and of enzyme diluted and assayed in the absence of albumin (○, □, △) are shown as a function of the final concentration of enzyme in the reaction mixtures. Enzyme was added to 0.20 ml reaction mixtures as 10 μl or 20 μl portions of appropriately diluted 105000g sediment (▲, △), KCl-solubilized enzyme (●, ○), or solubilized enzyme precipitated with $(NH_4)_2SO_4$ and dialysed against Buffer A (■, □). In each case the specific activity of enzyme assayed at about 30 μunits/ml of reaction mixture in the presence of albumin was set equal to unity.

activity from the walls of the tubes by washing with 10mg of albumin/ml of Buffer A were unsuccessful. The loss of activity in 10mM-potassium phosphate/ 1mM-MgCl$_2$/0.1mM-EDTA was the same at pH7.2 and pH5.2.

All proteins tested protected the enzyme against this inactivation. The specific activity of KCl-solubilized enzyme diluted in Buffer A containing 10mg of 'inert' protein/ml and assayed in reaction mixtures containing no other protein (so that the final concentration of inert protein in the reaction mixtures was 0.9mg/ml) was increased 2.5-fold by albumin or cytochrome c, 2.3-fold by myoglobin and 2.0-fold by ovalbumin or γ-globulin compared with the specific activity of enzyme diluted in Buffer A alone.

A final concentration of 2.5mg of albumin/ml in the reaction mixtures was sufficient to obtain maximum activity. Thus on addition of 5μg of KCl-solubilized enzyme protein in Buffer A alone to 0.20ml of reaction mixture containing 2.0, 0.5, 0.05 and 0mg of albumin, rates of 3.0, 3.2, 2.7 and 1.7pmol of cyclic AMP/min were obtained.

Consequently, in the experiments described in the present paper (unless stated otherwise) cyclic AMP phosphodiesterase activity was determined in the presence of 2.5mg of albumin/ml and enzyme samples were diluted, when necessary, with 10mg of albumin/ml of Buffer A. Previously (Londesborough, 1976b) enzyme assays were made in the absence of albumin, but as close as possible to a standard enzyme concentration (which corresponded to about 25μunits in Fig. 1) and the specific activity was corrected to that at the standard concentration by use of a calibration curve, essentially the same as the open symbols in Fig. 1. Results were similar to those reported here, except that recoveries of activity were generally lower, and in special cases (e.g. after gel filtration in the presence of 0.3M-KCl) much lower (J. Londesborough, unpublished work).

Subcellular distribution of low-K_m cyclic AMP phosphodiesterase activity

The distribution of cyclic AMP phosphodiesterase activity at 0.25μM-cyclic AMP among fractions obtained by differential centrifugation of yeast homogenates prepared in Buffer A/0.6M-mannitol is shown in Table 1. The activities are higher than those previously obtained by direct measurement of the rates of disappearance of 0.2μM-cyclic AMP from reaction mixtures containing no albumin and about 15μunits of cyclic AMP phosphodiesterase/ml (Londesborough, 1975). The difference is mainly caused by the presence of the albumin, because at this enzyme concentration the specific activity in the absence of albumin is only about 50% of its maximum value (Fig. 1).

With the present assay conditions, 90% of the whole homogenate activity was recovered after fractionation, and about 55% of the recovered activity was found in the 105000g sediment.

In these experiments the whole homogenate and 105000g supernatant were briefly dialysed (2–3h) against Buffer A before assay, and their activities increased by 70% during this dialysis. Addition to the assay mixtures of boiled 105000g supernatant at concentrations similar to those used in activity determinations caused about 30% inhibition of the cyclic AMP phosphodiesterase activity in both the 105000g sediment and the 105000g supernatant. This inhibition has not been further investigated.

In a few experiments the supernatant after removal of the 12000g sediment was dialysed against Buffer A or 10mM-potassium phosphate/1mM-MgCl$_2$/0.1mM-EDTA, pH5.7, before centrifugation at 105000g. This increased the amount of protein in the 105000g sediment by about 15%, but did not increase the amount of cyclic AMP phosphodiesterase activity in this sediment.

Dissociation of enzyme from the 105000g sediment

Dissociation of cyclic AMP phosphodiesterase I from the 105000g sediment was increased by increasing the salt concentration at pH7.2, or by increasing the pH in the presence of 0.1M-KCl (Figs. 2 and 3). At pH7.2, NaCl and KCl had identical effects, but MgCl$_2$ caused more dissociation than did KCl at the same ionic strength. However, at least at low concentrations of univalent cations, bivalent metal ion appeared to be essential for binding of the enzyme by the particles. Thus substitution of 2mM-EDTA for the 1mM-MgCl$_2$ normally present in Buffer A decreased the proportion of bound activity from 92% to 38% (Fig. 2). This effect of EDTA, in the absence of added MgCl$_2$, was also apparent in the presence of 10mM-KCl, but at 0.15M-KCl the amount of binding was the same in the presence of 1mM-EDTA/no MgCl$_2$ or 1mM-MgCl$_2$/0.1mM-EDTA. At 0.1M-KCl, binding was decreased by the addition of 15mM-EDTA (not shown), but it was not clear that this was due to chelation of bivalent metal ions, because the size of the effect was close to that expected from the large but uncertain increment in ionic strength caused by the quadrivalent EDTA anion.

In 0.1M-KCl, about 95% of the enzyme was bound at pH4.7 (Fig. 3). Below pH4.2 the enzyme was irreversibly inactivated. Within experimental error, the extent of binding decreased linearly with pH above pH4.7, and was 30% at pH9.3. At the higher pH values, MgCl$_2$ was omitted from the buffers, because it caused a precipitate, 20mM-Tris/HCl was included to control the pH, and the concentration of KCl was lowered to 90mM to maintain an ionic
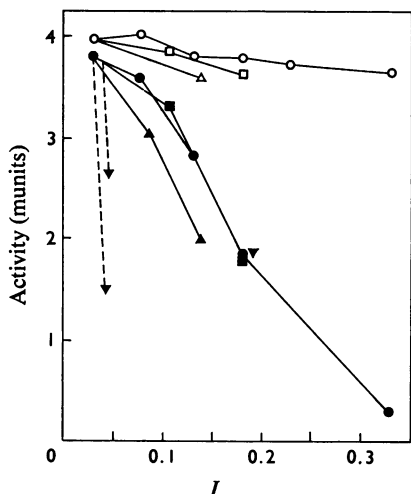
Fig. 2. *Effect of salt concentration on the dissociation of particle-bound cyclic AMP phosphodiesterase 1*
Portions of the 105000g sediment containing 21 mg of protein were suspended in 6.5 ml of Buffer A containing KCl (○, ●), NaCl (□, ■) or MgCl$_2$ (△, ▲) and centrifuged as described in the Materials and Methods section. The sedimented activity (●, ■, ▲) and total recovered activity (○, □, △) are shown. The sedimented activity is also shown when the 1 mM-MgCl$_2$ in the Buffer A was replaced by 1 mM-EDTA in the presence of 10 mM-KCl or 0.15 M-KCl, or by 2 mM-EDTA in the absence of neutral salt (▼).
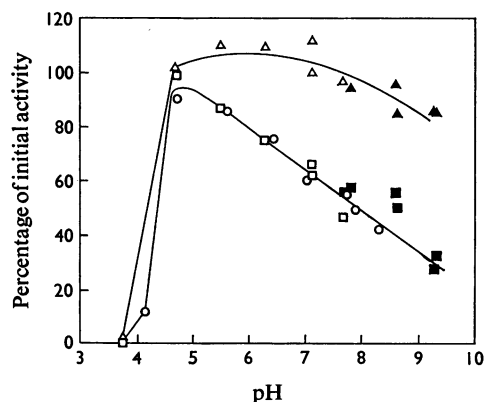

Fig. 3. *Effect of pH on the dissociation of particle-bound cyclic AMP phosphodiesterase*
Portions of the 105000g sediment containing 23 mg of protein were suspended in 6.7 ml of 10 mM-potassium phosphate / 1 mM-MgCl$_2$ / 0.1 mM-EDTA / 0.1 M-KCl, pH 3.6–8.2 (□, △), or of 10 mM-dipotassium phosphate / 0.1 mM-EDTA / 90 mM-KCl / 20 mM-Tris / HCl, pH 7.9–9.8 (■, ▲), and centrifuged as described in the Materials and Methods section. The percentages of the initial activity recovered in the supernatant plus sediment (△, ▲) and in the sediment alone (□, ■) are plotted against the pH of the suspensions. Results are also shown of a similar experiment in which portions (containing 4.0 munits of enzyme and 20 mg of protein in 7.0 ml of buffer) of the sediment collected between 34000g and 105000g were used (○).

strength of 0.13 g-ions/litre. Above pH 7.5, the activity in the supernatants decreased by about 25% during overnight storage at −25°C. This instability is probably responsible for the decline in recovery of total activity above pH 7.5.

In contrast with the sensitivity of the binding to pH and ionic strength, the binding was independent of temperature between 5 and 20°C. Thus for 105000g sediment suspended in Buffer A/0.1 M-KCl to a concentration of 2.6 mg of protein/ml, the bound activity was 48±2% of the total when duplicate samples were incubated for 40 min on ice and then centrifuged at 5°C, and 51±3% when duplicate samples were so incubated at 20°C and centrifuged at 21°C.

When tested in Buffer A/0.1 M-KCl containing 2.7 mg of 105000g sediment protein/ml, 1 mM-cyclic AMP, 1 mM-cyclic GMP, 20 mM-theophylline, 5 mM-glucose 6-phosphate, 2 mM-EGTA and 7 mM-mercaptoethanol had no effect on the extent of binding (55±3%). However, 10 mg of albumin/ml decreased the binding to 42% from 55%.

In these experiments the 105000g sediment was suspended in the appropriate buffer to a concen-

tration about tenfold smaller than the 'concentration' of the microsomal fraction *in vivo* (see below), and centrifuged (105000 g, 60 min) after equilibration for 20–30 min. At least at pH 7.2, dissociation of enzyme from the particles had reached a constant value after this time. Thus with 4.5 mg of 105000g sediment protein/ml of Buffer A containing 0.15 M-KCl or 0.1 M-KCl, the amount of sedimented activity (34% and 65% respectively) did not change when the equilibration time was varied between 7 min and 2 h. However, the extent of binding increased markedly with the concentration of the 105000g sediment (Fig. 4). Since 1 g of pressed yeast (i.e. 0.77 g of yeast cells with a dry weight of about 35%) provides 26 mg of Buffer A-washed 105000g sediment protein (Table 1), the minimum concentration of this material in the yeast cell is about 38 mg of protein/ml. In Buffer A/0.1 M-KCl (I 0.13) more than 80% of the enzyme was particle-bound at 6 mg of protein/ml, and in 20 mM-potassium phosphate / 0.2 M-KCl / 1 mM-MgCl$_2$ / 0.1 mM-EDTA (I 0.26) about 35% of the enzyme was bound at 23 mg of protein/ml. The intracellular ionic strength of yeast is not accurately known, but probably lies
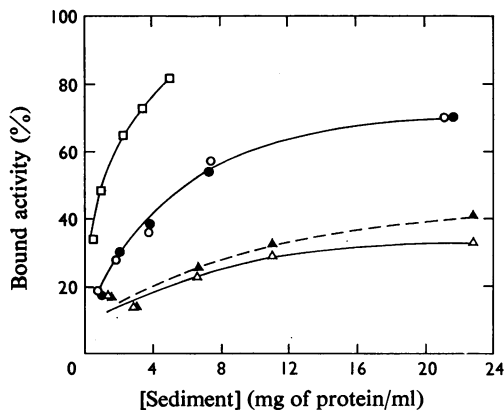
Fig. 4. *Effect of sediment concentration on the dissociation of particle-bound cyclic AMP phosphodiesterase*

The percentage of bound activity is shown when the 105000g sediment was suspended to the indicated concentration in Buffer A/0.1M-KCl (I0.13) ($\square$), 10mM-potassium phosphate/1mM-MgCl$_2$/0.1mM-EDTA/0.15M-KCl (I0.18), pH6.1 ($\bullet$) or pH7.2 ($\circ$), or in 20mM-potassium phosphate/1mM-MgCl$_2$/0.1mM-EDTA/0.2M-KCl (I0.26), pH7.2 ($\triangle$), and centrifuged as described in the Materials and Methods section. For the experiment at I0.26, the apparent amounts of sedimented activity before correction for the amount of supernatant trapped in the sediments are also shown ($\blacktriangle$).
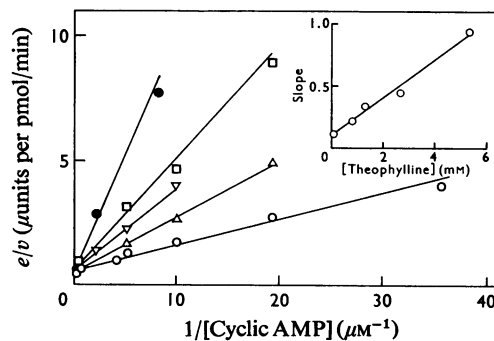

Fig. 5. *Inhibition of KCl-solubilized cyclic AMP phosphodiesterase by theophylline*

Initial velocities were measured in standard reaction mixtures containing 5.3 ($\bullet$), 2.64 ($\square$), 1.32 ($\triangledown$) and 0.80 ($\triangle$)mM-theophylline, or no theophylline ($\circ$), and the indicated amounts of cyclic AMP. In the inset the slopes of the double-reciprocal plots are plotted against the concentration of theophylline.

between these values (see the Discussion section). Results obtained at 0.15M-KCl (I0.18) are also shown, and it is noteworthy that, in contrast with the result at 0.1M-KCl reported in Fig. 3, the extent of binding under these conditions was the same at pH6.0 and pH7.2.

The large effect of concentration on the dissociation of enzyme from the 105000g sediment suggested that particle-bound stock enzyme might dissociate on dilution into reaction mixtures, despite their relatively low ionic strength (approx. I0.05). This was tested by suspending the 105000g sediment to a concentration of 50μunits/ml in an imitation reaction mixture consisting of 0.1M-Tris/HCl, pH8.1 at 5°C, containing 5mM-MgCl$_2$, 0.5mM-EDTA and 2.5mg of albumin/ml, and centrifuging the suspension at 5°C for 1h at 105000g. In duplicate experiments, 75±3% of the original activity was recovered in the supernatants, and 25±1% in the sediments.

Catalytic properties

In 0.1M-Tris/HCl, pH8.1, containing 5mM-MgCl$_2$ and 2.5mg of albumin/ml, KCl-solubilized cyclic AMP phosphodiesterase I had a K_m of 0.17μM, and was inhibited by theophylline in a simple

competitive manner with a K_i of 0.7mM (Fig. 5). In the absence of albumin, the K_m gradually decreased from 0.16 to 0.06μM as the enzyme concentration was decreased from 50 to 5μunits/ml (results not shown). However, at the lower enzyme concentrations the double-reciprocal plots were curved away from the 1/s axis at cyclic AMP concentrations below about 1μM, probably indicating that the instability of the enzyme in the absence of albumin increased at lower cyclic AMP concentrations. Evidently albumin did not cause an apparent increase of the K_m by sequestering significant amounts of cyclic AMP. At 0.25μM-cyclic AMP, 2.7mM-caffeine and 0.5mM-papaverine, each caused 30% inhibition, compared with 50% by 2.7mM-theophylline. Higher concentrations of papaverine could not be obtained.

At 2.5mM-MgCl$_2$ and 0.25μM-cyclic AMP, pH7.2 or 8.1, the rate of reaction in the absence of albumin did not change when the concentration of Tris/HCl was decreased to 20mM, and increased by about 30% when KCl was added up to 0.25M. This was true both for KCl-solubilized and particle-bound stock enzyme preparations. At 2.5mM-MgCl$_2$, 0.25μM-cyclic AMP, in the presence or absence of albumin, and with 0.1M-Tris/HCl or Tris/acetic acid buffers, the ·pH optimum for both KCl-solubilized and particle-bound enzymes was between pH7.5 and pH8.1. At pH5.2 and pH9.5 the initial rates were 17% and 40% respectively of that at pH8.1.

With particle-bound enzyme that had been dialysed for 16h against two changes of a 130-fold excess of 10mM-potassium phosphate, pH7.2, the initial rate at 0.25μM-cyclic AMP, pH8.1, in the absence of
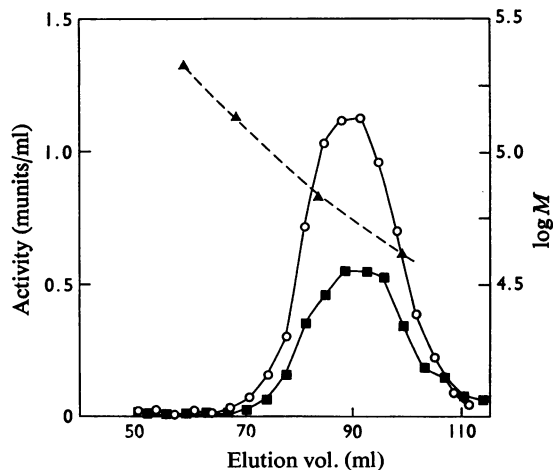
Fig. 6. Gel filtration of KCl-solubilized enzyme on Sephadex G-100 in Buffer A containing 0.5 M-KCl
Samples of KCl-solubilized enzyme, concentrated by $(NH_4)_2SO_4$ precipitation and dialysed first against Buffer A alone and then against Buffer A/0.5 M-KCl, were applied to a column (2.5 cm × 39 cm) of Sephadex G-100 and eluted with Buffer A/0.5 M-KCl at a flow of 40 ± 2 ml/h. A calibration curve (▲) of log(molecular weight) against elution volume was constructed at the same flow rate by using these standard proteins (apparent molecular weights in parentheses): ox γ-globulins (205000), pig heart lactate dehydrogenase (135000), ox serum albumin (68000) and ovalbumin (41000). The activity in the eluate after application of 25.4 munits of enzyme in 2.4 ml (○) and the activity (×20) after application of 0.80 munit in 1.5 ml (■) are shown.

albumin, was the same at 10 mM- or 1 mM-$MgCl_2$, 20% less at 0.1 mM-$MgCl_2$, and 50% less in the absence of added bivalent metal ion. Even with reaction mixtures treated with Chelex 100, the rate of reaction in the absence of added bivalent metal ions was 25–50% of that at 1 mM-$MgCl_2$. However, reaction was completely abolished by the addition of 30 μM-EDTA. Neither 1 mM-$MnCl_2$ nor 1 mM-$CaCl_2$ increased the reaction rate in the absence of Mg^{2+}, and at 10 mM both salts apparently inhibited the reaction by about 40%.

The following compounds altered the initial rates by less than ±15% when individually added to standard reaction mixtures containing 60 or 12 μunits of particle-bound enzyme/ml and no albumin; 0.1 mM- and 1.0 mM-NADH and -NADPH, 0.25 mM- and 2.5 mM-NAD⁺, -NADP⁺, -CoA, -acetyl-CoA, and -UDP-glucose, 0.5 and 5.0 mM-ATP, -phosphoenolpyruvate, -pyruvate, -glycerol 1-phosphate and -fructose 6-phosphate. Glucose 6-phosphate at 0.5 and 5.0 mM caused an activation of about 25% at

the lower enzyme concentration, but not at the higher. The product of the hydrolysis of cyclic AMP, 5'-AMP, did not inhibit at 60 or 400 μM. The 3':5'-cyclic nucleotides cyclic GMP, cyclic UMP and cyclic IMP did not alter the rate of reaction when present at 35 or 350 μM. Failure of these very large excesses (140- and 1400-fold) of cyclic nucleotides to inhibit the hydrolysis of 0.25 μM-cyclic AMP indicates that they are not themselves substrates for cyclic AMP phosphodiesterase I.

Gel-filtration behaviour of KCl-solubilized enzyme

In Buffer A containing 0.5 M-KCl, the elution volume of cyclic AMP phosphodiesterase I from Sephadex G-100 was independent of sample concentration over a 20-fold range and independent of the amount of applied activity over a 32-fold range (Fig. 6). The elution volume corresponded to an apparent mol.wt. of about 55000. The single peak of activity eluted contained 94% and 82% of the applied activity at the higher and lower concentrations respectively.

In Buffer A alone, the elution volume decreased when samples containing more enzyme were used (Fig. 7). When 40.4 munits of enzyme were applied, the peak of the eluted activity corresponded to an apparent mol.wt. of at least 200000, but the profile of eluted activity was skewed towards lower molecular weights. About 90% of the applied activity was recovered. With 0.83 munit of enzyme applied, only 30% of the applied activity was recovered, mainly in a peak with an apparent mol.wt. of 40000. However, a smaller amount of activity was always recovered at a much smaller elution volume with an apparent mol.wt. of about 200000. With 12.5 munits of enzyme applied, the enzyme was eluted with an apparent mol.wt. of 89000, and again a shoulder at about 200000 was observed. Over 95% of the enzyme activity was recovered.

The decrease of apparent molecular weight with decrease in the amount of sample observed in Buffer A is not primarily due to the decreased concentration of inert protein. Thus when 1.1 munits of enzyme were applied to and eluted from a column equilibrated with Buffer A containing 5 mg of albumin and 1 mg of ovalbumin/ml (the ovalbumin at least being small enough to penetrate the same column volume available to the cyclic AMP phosphodiesterase), the enzyme was eluted with an apparent mol.wt. of 48000 (Fig. 7). However, 54% of the applied activity was recovered, compared with 30% when 0.83 munit of enzyme was applied in the absence of inert protein.

The Buffer A/0.5 M-KCl-equilibrated column used in the experiments of Fig. 6 was somewhat larger than the Buffer A-equilibrated column of Fig. 7 (they had Blue Dextran 2000 exclusion volumes of 55 and
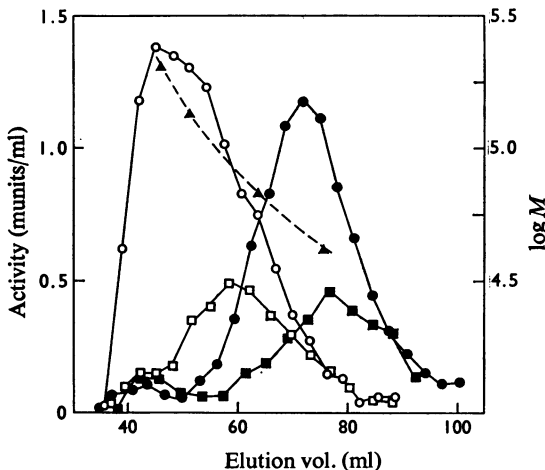
Fig. 7. *Gel filtration of KCl-solubilized enzyme in Buffer A*
A column (2.5 cm × 35 cm) of Sephadex G-100 was calibrated (▲) in Buffer A as described in the legend of Fig. 6, and concentrated and dialysed samples of KCl-solubilized enzyme in Buffer A were eluted at a flow rate of 38 ± 4 ml/h. The eluted activity after application of 40.4 munits in 4.0 ml (○) and 12.5 munits in 1.5 ml (□) and the activity (×20) after application of 0.83 munit in 1.5 ml (■) and 1.1 munits in 1.5 ml (●) are shown. In the latter case (●), the column was pre-equilibrated and the enzyme was eluted with Buffer A containing 5 mg of albumin and 1 mg of ovalbumin/ml, and the enzyme sample was dissolved in Buffer A containing 8 mg of albumin and 1 mg of ovalbumin/ml.

43 ml respectively). This does not seem to be important, because in another series of experiments (results not shown) 14.8 munits of enzyme was eluted from a Sephadex G-100 column equilibrated with Buffer A with an apparent mol.wt. of 140000, but after re-equilibration of the same column with Buffer A/0.3 M-KCl 9.7 munits of enzyme was eluted with an apparent mol.wt. of 45000.

Discussion

The simplest, and most likely, explanation of the gel-filtration behaviour of KCl-solubilized cyclic AMP phosphodiesterase I is that the enzyme contains a catalytically active subunit with a mol.wt. of about 50000, which, at low ionic strength and high enzyme concentration, either self-associates or binds to a larger, catalytically inactive protein. At the enzyme concentrations used in reaction mixtures (less than 60 μunits/ml) the enzyme would probably be present as the low-molecular-weight subunit, and any regulatory properties associated with the high-molecular-weight form would not be observed. However, because of the finding that the enzyme is inactivated by glass, the possibility that it also inter-

acts (hydrophobically?) with Sephadex, especially at high salt concentrations, must be considered, and the changes in apparent molecular weight need to be confirmed by an independent method.

The large inactivation occurring when the enzyme was diluted and assayed in the absence of albumin seems to be due to adsorption by glass. The best evidence for this was that the inactivation increased when samples were transferred to fresh glass tubes, and was complete when samples were incubated in the presence of small glass beads. The amount of inactivation was rather surprising, because the protein concentrations were by no means small in comparison with the concentrations of highly purified enzymes frequently used. Thus the lowest concentration of solubilized enzyme in the reaction mixtures of Fig. 1 was still 3 μg/ml. However, the physical conditions of a small reaction volume in a relatively large tube expose the enzyme to a large surface area, and it is also possible that proteins eluted from membranes are particularly prone to adsorption.

The very similar catalytic properties of particle-bound and KCl-solubilized enzyme with respect to the variation of specific activity with enzyme concentration in the absence of albumin, pH optimum, effect of ionic strength and K_m [i.e. 0.17 μM reported here for KCl-solubilized enzyme, and 0.12 μM reported by Londesborough (1975) for particle-bound enzyme] suggest either that most of the particle-bound enzyme rapidly dissociates when samples are diluted into reaction mixtures, or that the catalytic properties of the soluble enzyme are not altered when it becomes particle-bound. Ultra-centrifugal examination showed that, at a concentration of 50 μunits/ml, 25% of the enzyme remained particle-bound in imitation reaction mixtures at equilibrium, but it cannot give information about the situation in the first few minutes after addition of enzyme to the reaction mixtures. Although no certain information is available about the catalytic properties of enzyme that is still particle-bound, it is not likely to be very much more active than the solubilized enzyme, because then the extra activity of residual particle-bound enzyme would have been observed in experiments with high concentrations of particle-bound stock enzyme (e.g. Fig. 1).

Apparently cyclic AMP phosphodiesterase I is highly specific for cyclic AMP, because it was not inhibited by very large excesses of cyclic GMP, cyclic UMP or cyclic IMP. Further, the 105000 g sediment hydrolyses cyclic GMP only slowly, if at all (S. Tikanoja & J. Londesborough, unpublished work). This was tested by substituting cyclic [³H]GMP for the cyclic [³H]AMP in the assay system described by Londesborough (1975), and isolating remaining cyclic GMP by t.l.c. The rate of disappearance of cyclic GMP was less than 5% of that of cyclic AMP.

The high-K_m, soluble, cyclic nucleotide phosphodiesterase of baker's yeast has roughly equal activities towards 1 mM-cyclic AMP and -cyclic GMP (Fujimoto et al., 1974). However, this enzyme has a K_m of about 100 μM for cyclic GMP between 500 μM and 5 μM (J. Londesborough, unpublished work), so that its activity towards physiological concentrations of cyclic GMP is presumably exceedingly small. A cyclic GMP phosphodiesterase with significant activity at physiological concentrations of cyclic GMP has yet to be detected in yeast.

The role of Mg^{2+} ions in the reaction is uncertain; although the enzyme was inhibited by 30 μM-EDTA, activity persisted in the absence of added $MgCl_2$. Possibly concentrations of bivalent metal ion (not necessarily Mg^{2+}) as low as those of cyclic AMP are sufficient. Mn^{2+} and Ca^{2+} appeared to inhibit the enzyme, and may account for the inhibition observed when boiled 105000 g supernatant was added to reaction mixtures. A number of coenzymes and metabolites were tested for possible modulatory effects on the enzyme's activity. The only effect found was a small activation by glucose 6-phosphate, and this is in the opposite direction to that required to explain the ninefold increase in intracellular cyclic AMP when glucose is added to resting yeast (Van der Plaat, 1974). As with most cyclic nucleotide phosphodiesterases, activity was inhibited by theophylline, caffeine and papaverine. The K_i for theophylline was 4000 times the K_m for cyclic AMP.

It is noteworthy that the bulk intracellular pH of yeast varies from pH 6.4 to 7.5, depending on the nutritional conditions (Kotyk, 1963), and over this pH range the activities of cyclic AMP phosphodiesterase I and yeast adenylate cyclase (pH optimum about 6.2; Londesborough & Nurminen, 1972; K. Varimo & J. Londesborough, unpublished work) change in opposite directions. After homogenization in the presence or absence of Lubrol PX, the baker's yeast used in the present work contains 14 or 28 munits respectively of adenylate cyclase/g of pressed yeast in the presence of 5 mM-$MnCl_2$ and 2 mM-ATP, pH 6.2 (calculated from values/g dry wt. in Varimo & Londesborough, 1976). At pH 8.1, in the presence of 5 mM-$MgCl_2$ and 0.25 μM-cyclic AMP, the yeast contains 11.5 munits of cyclic AMP phosphodiesterase activity/g pressed weight, of which at least 5.5 munits is due to cyclic AMP phosphodiesterase I (Table 1). These activities become equal at about pH 7.3, which is consistent with an intracellular cyclic AMP concentration of about 0.25 μM. Concentrations between 0.01 and 0.6 μM have been reported (Van Wijk & Konijn, 1971; Sy & Richter, 1972; Schlanderer & Dellweg, 1974). In the presence of 0.1 M-KCl, and probably under the conditions of Van der Plaat (1974) mentioned above, addition of glucose to resting yeast results in a sharp fall in the intracellular pH from 7.5 to 6.4 (Kotyk, 1963). The

concentration of cyclic AMP would then be expected to increase until the cyclic AMP phosphodiesterase activity is again equal to that of adenylate cyclase. The increase may be quite large, because, at least at pH 8.1, the Michaelis constants of the low-K_m phosphodiesterases of yeast are less than 0.2 μM, so that above this concentration of cyclic AMP a relatively large increase in substrate concentration is required to increase the rate of reaction. Although this agreement is very satisfactory, it must be pointed out that the actual activity of adenylate cyclase in vivo is uncertain, because the available concentration of essential Mn^{2+} ions is unknown, and the activity of cyclic AMP phosphodiesterase in vivo is also uncertain, because this enzyme is probably largely particle-bound in vivo.

The bulk intracellular ionic strength of yeast is unknown. Normal concentrations of K^+, bicarbonate and orthophosphate ions are about 90 mM, 100 mM and 15 mM respectively (Rothstein & Demis, 1953; Gancedo & Gancedo, 1973), so that the physiological ionic strength probably does not greatly exceed that of animal tissues (I 0.15). At this ionic strength, and neutral pH, cyclic AMP phosphodiesterase I is largely dissociated from dilute suspensions of the 105000 g sediment, but the proportion of bound activity increases as the concentration of the sediment is increased towards the value in vivo, about 38 mg of protein/ml (Fig. 4). At pH 7.2, and 23 mg of sediment protein/ml, 70% of the enzyme was particle-bound at I 0.18 and 35% at I 0.26. Depending on the exact value of the bulk intracellular ionic strength of yeast, between 40 and 80% of cyclic AMP phosphodiesterase I is therefore expected to be particle-bound in vivo. Fig. 4 indicates the magnitude of possible artifacts in the distribution of enzymes between cytosol and particulate fractions if cells are disrupted and fractionated in buffers of physiological ionic strength, for the cell contents are almost invariably diluted five- to ten-fold during such procedures.

Scatchard (1949) plots of the data of Fig. 4 at I 0.13 and 0.18 were linear within experimental error (results not shown), and indicated maximum binding capacities of 0.22 and 0.19 munit/mg of sediment protein respectively. These values agree well with the specific activity of 0.19 munit/mg reported for Buffer A-washed 105000 g sediment in Table 1. The amount of activity sedimented from yeast homogenates was not increased by dialysis against 10 mM-potassium phosphate/1 mM-$MgCl_2$/0.1 mM-EDTA, pH 7.2 or 5.7. It therefore seems likely that the low-K_m cyclic AMP phosphodiesterase activity in the 105000 g supernatant of yeast homogenates prepared in Buffer A/0.6 M-mannitol is due to the same protein as cyclic AMP phosphodiesterase I, and remains soluble because the available particulate binding sites are saturated. This soluble low-K_m

activity has a K_m of about $0.2\,\mu$M and can bind to the $105000g$ sediment if cyclic AMP phosphodiesterase I is first removed by treatment with 0.3 M-KCl (Londesborough, 1975), and its apparent molecular weight during gel filtration on Sephadex G-100 varies with the concentration of KCl in the same way as does that of cyclic AMP phosphodiesterase I (J. Londesborough, unpublished work).

The effects of ionic strength and pH on the binding of cyclic AMP phosphodiesterase I (Figs. 2 and 3) are similar to their effects on the binding to membranes of lactate dehydrogenase (Melnick & Hultin, 1970), aldolase (Clarke & Masters, 1972) and glyceraldehyde phosphate dehydrogenase (Kant & Steck, 1973), and suggest that electrostatic forces are primarily responsible for the binding. The insensitivity of the binding to temperature is also consistent with electrostatic rather than hydrophobic interactions. In contrast with glyceraldehyde phosphate dehydrogenase (see, e.g., Letko & Bohnensack, 1974), the binding of cyclic AMP phosphodiesterase was unaffected by the presence of its substrate, or by high concentrations of the competitive inhibitor theophylline. However, free bivalent metal ion appeared to be essential for binding, at least at low concentrations of KCl (Fig. 2).

The $105000g$ sediment of yeast contains several different kinds of intracellular membranes as well as fragments of the plasma membrane. It is of interest to know which of these membranes bind cyclic AMP phosphodiesterase. RNA of the rough endoplasmic reticulum is probably not involved, because removal of 95% of the RNA from the $105000g$ sediment by treatment with ribonuclease did not cause dissociation of the enzyme (J. Londesborough, unpublished work). Further investigation of the nature of the binding sites can probably be best approached by studying the reassociation of the KCl-solubilized enzyme with the particulate fraction.

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