A Manganese-Dependent Adenyl Cyclase in Baker's Yeast, *Saccharomyces cerevisiae*

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Evidence exists that cyclic 3′,5′-AMP may be involved in the metabolic control of yeast, as has already been well documented for bacteria.[1] In *Saccharomyces carlsbergensis* the concentration of a bacterial attractant, probably cyclic AMP, depends upon the growth conditions,[2] and cyclic AMP, and several other nucleotides, prevent the inhibition by glucose of respiratory adaptation by *Sacch. cerevisiae*.[3] However, adenyl cyclase, the only enzyme known to synthesise cyclic AMP, has not been previously demonstrated in these yeasts. Recently Sy and Richter [4] reported a Mg-dependent cyclase in *Sacch. fragilis*, but were unable to detect activity in *Sacch. cerevisiae* and *Sacch. carlsbergensis*. In the present communication a particulate, Mn-dependent adenyl cyclase in *Sacch. cerevisiae* is reported.

Experimental. Commercial baker's yeast from the Rajamäki Factories of the Finnish State Alcohol Monopoly was used. Cells were disintegrated and cell envelopes prepared essentially as described by Nurminen *et al.*[5] except that the Tris-HCl buffer (pH 7.2) contained 0.3 mM EDTA, 7 mM mercaptoethanol and, usually, 1 mM $MnCl_2$, and Ballotini beads of 1 mm diameter were used. Adenyl cyclase was determined in reaction mixtures containing 5 mM $MnCl_2$, 1 mM $MgCl_2$, 10 mM NaF, 10 mM phosphoenolpyruvate, 0.25 mg/ml pyruvate kinase, 2 mM 8-^{14}C-ATP (1800 cpm/nmol), and 0.4 mM 3′,5′-cyclic AMP. Unless otherwise stated the mixture was buffered to pH 6.8 with 100 mM HEPES-KOH.[6] Aliquots of 0.1 ml were withdrawn at suitable intervals, added to 0.1 ml of 1 mM cyclic AMP in 75 % ethanol, heated for 1 min at 97°C, and centrifuged. Cyclic AMP was isolated from the supernatants by TLC on tetraborate impregnated silica, developed first with 50 % ethanol and then in the opposite direction with water saturated 1-butanol.[7] The cyclic AMP spots were scraped off, suspended in 10 ml of scintillant (60 g naphthalene, 4 g PPO, 0.2 g dimethyl-POPOP in dioxane to 1 liter) and counted in a Packard Tri-Carb 2420. On rechromatography after treatment with beef heart phosphodiesterase more than 80 % of the radioactivity originally found in cyclic AMP migrated as 5′-AMP.

Other enzymic activities were determined as previously described.[5] Protein was measured according to Racusen and Johnston.[8] Radioactive ATP was from New England Nuclear (NEN Chemicals GmbH, Frankfurt a.M., Germany) and other biochemicals from Boehringer GmbH Mannheim, Germany.

Very little new cyclic AMP was formed when whole homogenates were incubated in the adenyl cyclase system in the absence of $MnCl_2$ and presence of 5 mM $MgCl_2$. The activity of a crude 10 min × 1000 *g* sediment was stimulated 9 fold by the addition of 5 mM $MnCl_2$ (Fig. 1). Higher

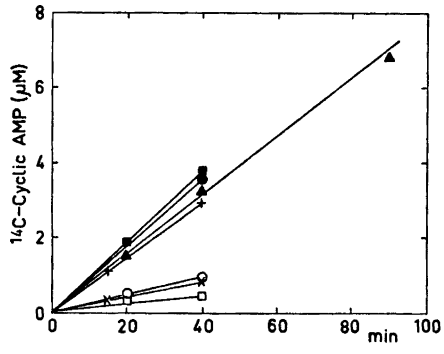

Fig. 1. Magnesium and manganese requirement of adenyl cyclase from *Sacch. cerevisiae*. Activity was measured as described under "Experimental" with the modifications indicated: 10 min × 1000 *g* sediment incubated at 7.7 mg/ml (with 40 mM Tris-HCl as buffer, pH 6.6) at final metal concentrations of 10.4 mM $MgCl_2$ (○), 5.4 mM $MgCl_2$ (□), 10 mM $MnCl_2$ plus 0.4 mM $MgCl_2$ (●), 5 mM $MnCl_2$ plus 0.4 mM $MgCl_2$ (■), and 5 mM $MnCl_2$ plus 5.4 mM $MgCl_2$ (▲); cell envelopes incubated at 1 mg/ml (with only 6.7 mM phosphoenolpyruvate) at 5 mM $MnCl_2$ alone (+), and 12.5 mM $MgCl_2$ alone (×).

concentrations of $MnCl_2$ caused little further effect, and the activity was slightly increased by lowering the concentration of $MgCl_2$ to 0.4 mM. In the

complete absence of $MgCl_2$ regeneration of ATP by pyruvate kinase is inefficient: nonetheless, as shown in Fig. 1 for a well washed envelope fraction (with relatively low ATPase), activity was 3.5 fold greater in 5 mM $MnCl_2$ alone than in 12.5 mM $MgCl_2$ alone. Under these conditions, adenyl cyclase activity with 12.5 mM $CoCl_2$ was about equal to that with 12.5 mM $MgCl_2$ and essentially no activity was found with 12.5 mM $CaCl_2$ or $ZnSO_4$.

The cyclase activity was proportional to the amount of cell envelopes up to a rate of synthesis of cyclic AMP of at least 0.3 μmol l^{-1} min^{-1}. With 10 min × 1000 *g* sediments or purified cell envelopes as enzyme source cyclic AMP production was linear for at least 90 min (Fig 1). However the presence of a powerful, mainly soluble, phosphodiesterase (*cf.* Speziali and Van Wijk [9]) necessitated shorter incubation times (less than 20 min) for *intra*cellular fractions and whole homogenates. No activity could be demonstrated with unbroken yeast cells. The results of Table 1 show that most of the total activity was particle bound, and 20 % to 35 % precipitated in the 10 min × 1000 *g* sediment depending on the divalent cation type and concentration in the medium. Washed cell envelopes prepared from 10 min × 1000 *g* sediments contained 12 % of the total cyclase activity, no hexokinase, less than 3 % of the total NADH oxidase and 10 % of the total oligomycin insensitive Mg-dependent ATPase, which is believed to be a marker for the yeast plasma membrane.[10-12] Further disintegration of isolated envelopes in the Mickle apparatus released the adenyl cyclase activity, which could then be recovered in the 60 min × 140 000 *g* precipitate.

Table 1. Distribution of adenyl cyclase among subcellular fractions of *Sacch. cerevisiae*. Homogenates were prepared and fractions assayed as described under "Experimental". The homogenisation medium contained: (a) 1 mM $MnCl_2$, and (b) 10 mM $MgCl_2$. Total activities are expressed as nmol min^{-1} g^{-1} fresh yeast and specific activities as pmol min^{-1} mg^{-1} protein.

Fraction	Total activity	Specific activity
(a)		
Whole homogenate	4.4	53
10 min × 1000 *g* sediment	1.5	42
10 min × 12 000 *g* sediment	1.0	200
10 min × 12 000 *g* supernatant	4.0	85
(b)		
10 min × 1000 *g* sediment	2.5	71
60 min × 140 000 *g* sediment	3.0	185
60 min × 140 000 *g* supernatant	1.7	67

The adenyl cyclase activity of both whole cells and cell envelopes was stimulated only 40 % by 10 mM NaF. With cell envelopes the apparent K_m for ATP was 2.9 mM at pH 6.8 in the presence of 0.4 mM cyclic AMP (Fig. 2), and an apparent pH optimum of 5.7 was observed in the presence of 2 mM ATP and 0.4 mM cyclic AMP (Fig. 3).

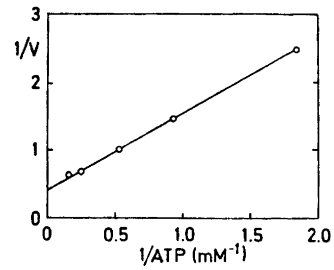

Fig. 2. Apparent Michaelis constant for ATP with adenyl cyclase of cell envelopes. Activity was measured as described under "Experimental" at appropriate ATP concentrations. The initial velocity, *V*, is arbitrarily set equal to unity at 2 mM ATP.

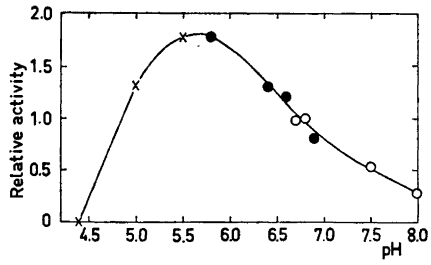

Fig. 3. Apparent pH optimum for adenyl cyclase of cell envelopes. Activity was measured as described under "Experimental" using the following buffers: 100 mM HEPES-KOH (○), 100 mM PIPES-KOH[6] (●), and 100 mM potassium acetate (×).

These results show the presence of adenyl cyclase in baker's yeast, *Sacch. cerevisiae*, which is clearly necessary if cyclic AMP is to exert a physiological control. The specific activity of the Mn-dependent cyclase in whole homogenates is about 20 times greater than that of the Mg-dependent cyclase in *Sacch. fragilis*.[4] Both enzymes have low pH optima and are only weakly stimulated by NaF, in contrast to many mammalian enzymes.[13] In *Sacch. fragilis* sphaeroplasts 80 % of the total activity can be located in the plasma membrane. Our results suggest a considerable part of the total activity is so located in *Sacch. cerevisiae*, and much of the activity found in other particulate fractions probably originates from the plasma membrane which is known to fragment during disruption of this hard-walled yeast, and also precipitate in these fractions.[5,11]

1. Pastan, I. and Perlman, R. *Science* **169** (1970) 339.
2. Van Wijk, R. and Konijn, T. M. *FEBS Lett.* **13** (1971) 184.
3. Fang, M. and Butow, R. A. *Biochem. Biophys. Res. Commun.* **41** (1970) 1579.
4. Sy, J. and Richter, D. *Biochemistry* **11** (1972) 2788.
5. Nurminen, T., Oura, E. and Suomalainen, H. *Biochem. J.* **116** (1970) 61.
6. Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S. and Singh, R. H. M. *Biochemistry* **5** (1966) 467.
7. Upton, J. D. *J. Chromatog.* **52** (1970) 169.
8. Racusen, D. and Johnstone, D. B. *Nature* **191** (1961) 492.
9. Speziali, G. A. G. and Van Wijk, R. *Biochim. Biophys. Acta* **235** (1971) 466.
10. Boulton, A. A. *Exptl. Cell Res.* **37** (1965) 343.
11. Matile, P., Moor, H. and Mühlethaler, K. *Arch. Mikrobiol.* **58** (1967) 201.
12. Nurminen, T., Oura, E. and Suomalainen, H. *Fed. Eur. Biochem. Soc. Meet., 5th, Prague 1968*, Abstr. Commun., p. 115.
13. Jost, J.-P. and Rickenberg, H. V. *Ann. Rev. Biochem.* **40** (1971) 741.

Received October 13, 1972.

Thiourea, an Effective Inhibitor of the Non-Enzymatic Ethanol Oxidation in Biological Extracts

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The determination of acetaldehyde in biological extracts is complicated by the formation of this compound in blood and tissue precipitates when ethanol is present.[1,2] This acetaldehyde is formed by a non-enzymatic oxidation of ethanol by means of a radical chain reaction mechanism. A semidehydroascorbate peroxy radical has been hypothesized to act as an electron acceptor in the reaction.[2] The reactive radicals are produced by the autoxidation of ascorbic acid. Since thiourea is an effective inhibitor of the latter reaction,[3-5] acting as a free radical acceptor and breaking the radical chain reaction,[6-8] it was thought that it should block the non-enzymatic oxidation of ethanol. The protective effect of thiourea can be looked upon as a type of antioxidation.[6,9]

It has now been found that the non-enzymatic ethanol oxidation in the supernatant of fresh liver homogenate precipitated by perchloric acid (PCA), is completely inhibited by 25 – 50 mM thiourea (Table 1). If the incubation time at 65°C was 15 – 20 min, and the homogenate concentration ≤ 20 % (w/v), a 25 mM thiourea concentration was enough to inhibit all ethanol oxidation in the sample. Even a 8 mM thiourea concentration was able to inhibit 80 – 90 % of the ethanol oxidation.

Thiourea was also added to samples containing 1.0 mM ascorbic acid and 20 mM ethanol and again was found inhibit non-enzymatic ethanol oxidation (Table 1). The fact that thiourea is effective in both liver extracts and in pure ascorbic acid solutions is strong evidence that ascorbic acid is involved in both reactions.[2]

An alternative explanation for the above results could be that thiourea interacts with the acetaldehyde present in the sample. In order to test this hypothesis thiourea was added to solutions containing acetaldehyde. As Table 2 illustrates, 100 mM thiourea had no effect on the acetal-