CYCLIC 3',5'-ADENOSINE MONOPHOSPHATE STIMULATES TREHALOSE DEGRADATION IN BAKER'S YEAST

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SUMMARY: The levels of cyclic 3',5'-AMP and trehalose, as well as the specific activity of the trehalase have been investigated in cells of baker's yeast (Saccharomyces cerevisiae) during the lag phase preceding growth. During the first few minutes a substantial increase in the intracellular concentration of cyclic 3',5'-AMP was observed, followed by a 6-8 fold increase in trehalase activity concomitant with the rapid degradation of trehalose. Cell free extracts prepared from resting yeast were shown to contain a cryptic trehalase, which under physiological conditions could be activated by cyclic 3',5'-AMP to the same degree as in vivo. These observations suggest that in the lag phase of growth, the level of trehalose in baker's yeast is under control of a system, regulated by the level of cyclic 3',5'-AMP.

INTRODUCTION

Little is known of the function(s) of cyclic 3',5'-AMP[1] in yeast cells. The most recent review on the physiology and biochemistry of yeasts (1) does not even mention its presence in yeast, which was first reported by Cheung (2). Recently the existence of a membrane-bound Mg-dependent adenyl cyclase and a Mn-stimulated cAMP phosphodiesterase in Saccharomyces fragilis have been reported (3,4). In contrast to S.fragilis the adenyl cyclase in S.cerevisiae is Mn-dependent (5). Several yeast strains appear to contain a cAMP binding protein, whose function is still unknown (6).

Attempts to elucidate the function of cAMP in yeast have been directed towards the control of glucose repression as this has been shown to exist in bacteria (7). In Saccharomyces carlsbergensis (8) as well as in S.fragilis (3) the intracellular level of cAMP appears to depend upon growth conditions and carbon source. Although the inhibition by glucose of respiratory adaptation by protoplasts of S.cerevisiae could be overcome by cAMP, several other nucleotides, such as AMP and ATP, were just as effective (9). There appears to be no evidence that the level of cAMP in yeast is correlated with the sensitivity of invertase synthesis to glucose repression (10). The function of cAMP in the control of yeast catabolism is therefore far from clear. As cAMP is involved in the control of reserve carbohydrate metabolism in other eukaryotes (11), we decided to investigate its possible relation to trehalose,

[1] Abbreviations: cAMP, cyclic 3',5'-adenosine monophosphate; deoxyglucose, 2-deoxy-β-D glucose; TCA, trichloroacetic acid; G-6-P, glucose-6-phosphate.

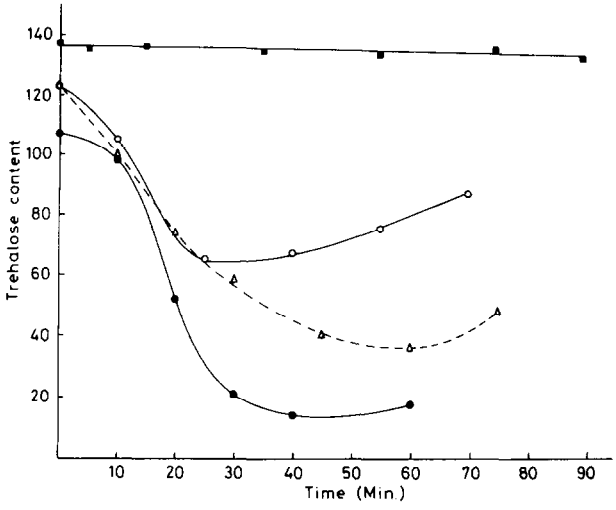

Fig.1. Trehalose degradation during fermentation by cells of baker's yeast. ●, complete medium; ○, nitrogen omitted; Δ, cycloheximide (5 μg/ml) added; ■, glucose substituted by deoxyglucose (0.12 M). Different batches of yeast were used.

a carbohydrate, which can be rapidly mobilized.

Requirements for the synthesis of trehalose by cells of baker's yeast have been described (12,13). In the lag phase of growth and during the budding-cycle trehalose is rapidly degraded (14,15). In this communication we provide evidence for the involvement of cAMP in the control of degradation of trehalose during the lag phase preceding growth.

MATERIALS AND METHODS

Yeast and incubation. Commercially grown baker's yeast (S.cerevisiae) from our factory was used. For incubation a modified Miller medium (16) containing 0.25 M glucose buffered with 0.17 M citrate pH 5.6 was used, which was rapidly inoculated with 7 g of yeast dry matter per litre. Incubation was at 28°C with slow stirring but without aeration. Samples were rapidly filtered over Selectron BA 85 filters (Schleicher und Schüll). The intracellular water content was determined from the inulin space, using inulin-[^{14}C]carboxylic acid (The Radiochemical Centre). Theophylline was from Brocades-ACF, cycloheximide from The Upjohn Co. and deoxyglucose from Baker Chemicals.

Extracts for cAMP and trehalose assay. The filter-mat containing about 100 mg (wet weight) yeast was immediately dropped into 2.0 ml of ice-cold 5 % $HClO_4$, containing 2.8 mM of LiCl. After freezing and thawing three times, the cells were removed by centrifugation and the supernatant neutralized with solid $KHCO_3$. After removal of perchlorate Li^+ was determined in an aliquot with a Perkin Elmer Atomic Absorption Meter to correct for water in the sample. In cases of low cAMP conc. 500 mg of yeast and 3.0 ml of $HClO_4$ were used.

For trehalose assay the yeast cake (100 mg) was washed twice with ice-cold distilled water and the filter-mat was dropped into 2 ml of ice-cold 10 % TCA and further processed as described by Trevelyan and Harrison (17), except that the total volume was reduced to 10 ml.

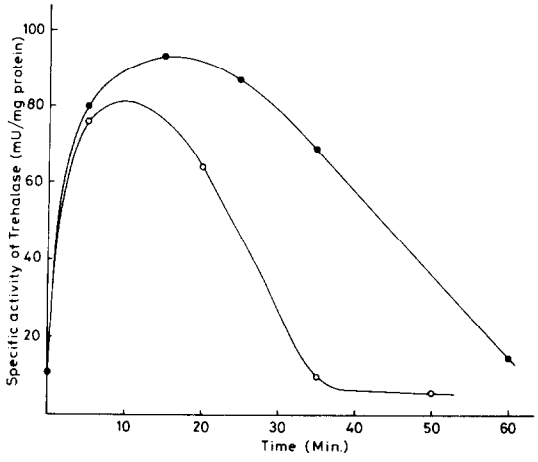

Fig.2. Specific activity of trehalase in extracts of cells at different stages of the fermentation.

●, complete medium; o, nitrogen omitted.

Cell free extracts for trehalase assay. The yeast cake (100 mg) was washed twice with ice-cold 50 mM phosphate buffer pH 7.0, containing 0.2 mM EDTA and resuspended in 1 ml of the same buffer in a conical bottom 15 ml centrifuge tube. 2.0 g Ballotini glass beads (Ø 0.25-0.30 mm) were added and the mixture was vigorously agitated on a Vortex mixer (Scientific Industries) for 3 min. at 4°C. From the resulting extract 0.5 ml was removed with a pipette and centrifuged at 1,200 x g for 15 min. at 4°C. Enzyme activity was assayed in the supernatant. Protein was estimated by the method of Lowry et al. (18).

Cell free extracts for trehalase activation. 25 g of pressed yeast was washed twice by centrifugation with 50 mM phosphate buffer pH 6.2, containing 0.6 mM EDTA. About 5 ml of this buffer was added to the sediment. To achieve cell disruption, the paste was pressed twice at -28°C in an X-press (Type X-25, AB Biox-Sweden). After thawing the extract was centrifuged at 15,000 x g for 30 min. at 4°C. The supernatant was kept on ice and used for activation.

Assay procedures.

Determination of cAMP. cAMP was estimated with the use of BDH Binding Protein according to the procedure supplied by the manufacturer (BDH Ltd, Poole, England), which is based on the assay of Brown et al.(19). Due to the presence of perchlorate, incubation time was prolonged to 150 min. [^{3}H]cAMP (27 Ci/mmol) was from The Radiochemical Centre, Amersham, U.K. Radioactivity was determined by liquid scintillation counting.

Determination of trehalose. Trehalose in TCA extracts was determined with the anthrone reagent (17). Trehalose content is expressed in mg carbohydrate (as hexose) per g yeast dry matter.

Determination of trehalase activity. The assay procedure of Küenzi and Fiechter (14) was used. Glucose, however, was determined enzymatically (20). No interference by other reducing substances was noted after suitable dilution. For each incubation, samples were routinely withdrawn at zero time, 20 and 40 min. A unit of trehalase converts 1 µmol trehalose per min. in the assay system. Glucose oxidase Grade I (less purified enzyme may contain appreciable amounts of trehalase) and Peroxidase Grade II were from Boehringer, Mannheim. Chromogen was from Worthington.

Activation of trehalase. 0.5 ml of the extract (usually containing 100-

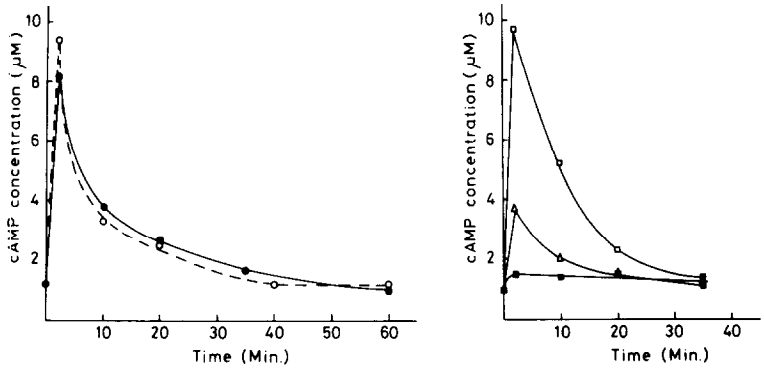

Fig.3. Intracellular cAMP concentration in cells of baker's yeast during fermentation.

Symbols as in fig.1; □, theophylline added (8 mM).

-130 mg protein per ml) was mixed with 50 µl of cAMP (or another compound to be tested) in 50 mM phosphate buffer pH 6.2 at 0°C. Activation was started by immersion of the tubes, containing the mixture in a thermostat-bath at 30.0°C. The reaction was stopped by addition of 5-7 ml of ice-cold phosphate buffer pH 7.0, containing 0.2 mM EDTA. The mixture was kept on ice until assayed for trehalase activity. cAMP was from Sigma, other nucleotides and G-6-P were from Boehringer, glucose was from BDH.

RESULTS

After inoculation of the yeast a rapid degradation of trehalose occurs, which is complete in about 45 min., as shown in fig.1. This confirms the findings of Panek (15). Omission of nitrogen or blocking of protein synthesis leads to less degradation and resumed synthesis. No degradation occurs when glucose is replaced by deoxyglucose. As shown in fig.2, trehalase activity in cell free extracts shows a pattern corresponding with the observed break down. Maximum activity coincides with the fastest break down, the decay being faster when nitrogen sources are omitted.

Fig.3 shows that the trehalase activity maximum is preceded by a cAMP concentration peak at about 2 min. after inoculation. This cAMP signal decays and returns to about the basic level when trehalose degradation has ceased. Decay of the signal could not be prevented by addition of theophylline (8 mM) to the medium. The fact, however, that no cAMP signal occurs and trehalose is not degraded when glucose is substituted by deoxyglucose leads us to believe that cAMP is involved in trehalase activation.

The first experiments to achieve activation *in vitro* were performed with Ballotini extracts. Preincubation at 30°C and pH 7.0 with 0.1 mM cAMP only stimulated trehalase activity by 50 %. As the conditions were very different from those *in vivo* we suspected a dependence on protein concentrat-

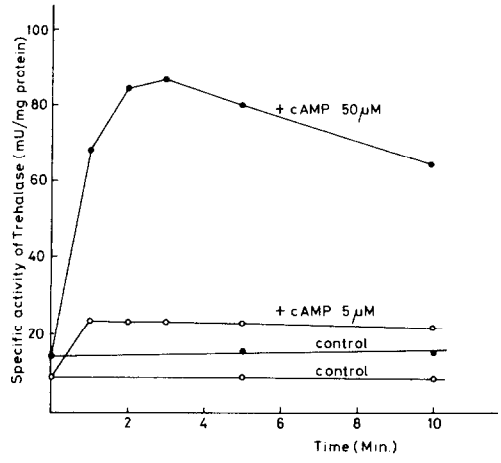

Fig.4. Activation rate of cell free extracts upon addition of different concentrations of cAMP.

ion. Subsequently we found that the protein concentration has to be at least 60 mg/ml. The pH was changed to 6.2, which appeared to be optimal and equal to the intracellular pH under our conditions of fermentation.

The trehalose content of different batches of commercial baker's yeast varies between 11 and 14 %. It was also observed that the endogenous trehalase activity varies (between 8 and 14) as also does the activating ability. The activation, as shown in table I, is under optimal conditions at least 5.6 times and usually between 6 and 8 fold. Activation under our conditions is very fast and the time course reflects the warming up of the reaction mixture (fig.4). No activation occurs when cAMP is omitted. Activation appears to decay at a rate comparable to conditions in vivo (fig.2). Addition of theophylline increases the activity after prolonged incubation (table I).

The activation of cryptic trehalase is specific for cAMP since other adenine nucleotides in physiological concentrations are ineffective (see table II). Glucose and G-6-P were tested for their action because earlier investigations (21) showed that the course of the intracellular G-6-P concentration resembles the cAMP signal. G-6-P concentration in resting cells is 0.1 mM and rises in 2 min. to 4.0 mM, after which it decreases in 30 min. to a steady level of 1.2 mM.

The degree of activation depends on the cAMP concentration, as illustrated in fig.5. The data fit a Lineweaver-Burk type of plot, giving an apparent K_a for half activation of 2.26 μM. Data obtained from resting cells fit well in this plot.

DISCUSSION

The data presented suggest that the break down of trehalose in the lag

Table I. Activating ability of different extracts.

Yeast batch	Protein conc. (mg/ml)	Incubation time (Min.)	Trehalase activity (mU/mg protein)	
			Control	Activated
110	95	3	8.3	70.6
189	91	3	14.4	86.4
269	106	3	9.1	52.5
269	106	10	9.1	40.3
269	106	10	9.1	51.0*

*Theophylline (8 mM) added.

Table II. Specificity of trehalase activation.

Addition	Concentration (mM)	Specific activity of trehalase (mU/mg protein)
None	-	8.9
cAMP	0.05	43.8
Glucose	110	8.2
G-6-P	20	10.1
AMP	1	10.7
ATP	4	9.4
Control, not incubated	-	9.1

phase of growth is regulated by the level of cAMP. This explanation for the observed utilization of trehalose differs from the suggestion of Panek (22), that the trehalase activity is regulated by ATP. Under our conditions, however, the ATP level is fairly constant (4 mM), during lag phase and subsequent growth (21). It is not likely that the activation reaction is a simple matter of equilibrium binding, since once activated the state of trehalase can be conserved by dilution and chilling.

Protein synthesis does not seem to be involved in the activation process, since in the presence of cycloheximide both trehalose degradation and the cAMP signal have been observed. Regulation of trehalase activity during the cell cycle involving protein synthesis has recently been suggested

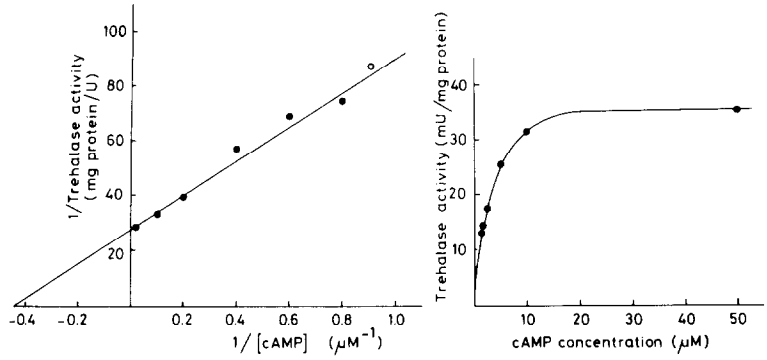

Fig.5. Dependence of trehalase activation in cell free extracts on cAMP concentration.

Incubation was for 1 min., protein conc. 108 mg/ml.

o, conditions in resting cells.

(14). For regulation of the level of trehalose not only activation, but also inactivation of trehalase may be important, as can be seen from figs 1 and 2. From these findings one might speculate that trehalase activity tends to be low at low cAMP level, its rate of inactivation, however, being influenced by the ability of the cell to grow.

The mechanism of activation is still unknown. It could very well be that due to the presence of glucose, the yeast adenyl cyclase is activated by intracellular glucose or an early metabolite, such as G-6-P. The subsequent enhanced level of cAMP leads to activation of cryptic trehalase. Preliminary experiments indicate that the presence of Mg-ATP is a requirement for activation, but as yet there is no evidence for the involvement of a protein kinase. Attempts are under way to purify the proteins involved and elucidate the activation mechanism.

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