Content of Cyclic 3′,5′-Adenosine Monophosphate and Adenylyl Cyclase in Yeast at Various Growth Conditions†

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ABSTRACT: A membrane-bound preparation of adenylyl cyclase was isolated from homogenates of *Sacchromyces fragilis*. In organisms growing in a maximally aerated culture with glucose or lactate, activity of the cyclase increased three- to fourfold when growth entered late logarithmic and stationary phases. At submaximal aeration, the lactate-grown cells had a seven- to eightfold greater cyclase activity as compared to those grown in glucose. Likewise, the 3′,5′-adenosine monophosphate (cAMP) content of *S. fragilis* was found to depend on growth conditions and the carbon source. Cells grown in 2% lactate or 1.5% glucose contained twice as much cAMP after reaching the stationary phase. In contrast, cells cultured in 10% glucose maintained a steady, relatively low concentration of cAMP during the whole growth period.

Cyclic 3′,5′-adenosine monophosphate[1] has been shown to control a variety of functions in prokaryotes as well as in eukaryotes (Pastan and Perlman, 1970; Robinson *et al.*, 1971). This control includes catabolite repression in *Escherichia coli*, differentiation in slime mold, and various hormonal responses in mammalian systems. Little is known, however, of the function(s) of this nucleotide in yeast cells. *In vitro*, cAMP was shown (Chance and Schoener, 1964; Cheung, 1966) to affect oscillatory cycles of NAD^+ reduction and oxidation in cell-free extracts. Measurement of the intracellular concentration of cAMP in *Saccharomyces carlbergensis* showed an increase in the level of cAMP when yeast was derepressed from glucose repression (Van Wijk and Konijn, 1970). A cAMP phosphodiesterase seems to have a regulating function on the level of intracellular cAMP (Speziali and Van Wijk, 1971). In the preceding paper (Sy and Richter, 1972), we reported on the isolation of a protein that specifically binds cAMP. This binding protein could not be related to a protein kinase, and so far no function has been established for it. To gather more information about the functioning of cAMP in yeast, we turned our attention to its metabolism. We report here that the yeast *Saccharomyces fragilis* contains a membrane-bound adenylyl cyclase with an activity strongly influenced by growth conditions which were also found to modify the intracellular cAMP content.

Experimental Section

Growth conditions were the same as reported in the preceding paper (Sy and Richter, 1972).

Preparation of Adenylyl Cyclase from S. fragilis. The cells were suspended in two volumes of buffer containing 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.25 mM EDTA, and 1 mM dithiothreitol (buffer A), and disrupted by passing them through a French press cell at 15,000 psi. In later experiments, 20 mM potassium phosphate buffer (pH 7.0) was used instead of Tris-HCl. A crude particulate fraction containing the activity was obtained by a 10-min spin at 1000*g*. It was washed once with buffer A and resuspended in one volume of the same buffer. Aliquots were stored in liquid nitrogen until used. In some experiments, spheroplasts were prepared and lysed by osmotic shock (Davies and Elvin, 1964; Rozijn and Tonino, 1964). Spheroplast membranes were obtained by centrifugation at 1000*g* for 15 min.

Assay for Adenylyl Cyclase Activity. The membrane fraction (200 μg of protein) was incubated at 30° for 20 min in a final volume of 0.1 ml containing: 20 mM potassium phosphate (pH 7.0), 5 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM P-enolpyruvate, 50 μg of pyruvate kinase; and 0.9 mM 8-[^{3}H]-ATP (27.5 μCi/μmole) or α-[^{32}P]ATP (22.5 Ci/mole). In addition, 0.5 mM of unlabeled cAMP was present as a trap (Bar and Hechter, 1969). The reaction was stopped by the addition of 0.1 ml of 40 mM ATP–12.5 mM cAMP as carrier, followed by heating for 5 min in a boiling-water bath. The cAMP formed was isolated following the method of Krishna *et al.* (1968). The boiled reaction mixture was diluted with 0.3 ml of H_2O, and the precipitated protein was centrifuged at 10,000*g* for 10 min. The supernatant fraction was then chromatographed on a Dowex 50W-X8 (H^+) column (0.5 × 4.0 cm). The column was washed with water and 0.5-ml fractions were collected. The cAMP-containing fractions were pooled and contaminating nucleotides were removed by barium sulfate precipitation (Krishna *et al.*, 1968). The precipitate was discarded and 2-ml aliquots of the supernatant fraction were either counted in 20 ml of Bray's scintillation fluid (Bray, 1960), or they were lyophilized and dissolved in a small amount of water. To identify the isolated radioactive product as cAMP, the water-soluble sample was chromatographed in the following thin-layer systems: (1) PEI-cellulose, 0.25 N LiCl; (2) cellulose, 1 M ammonium acetate–95% ethanol (30:75); (3) cellulose, isopropyl alcohol–concentrated NH_4OH–0.1 M H_3BO_3 (60:10:30). In all cases, the radioactive product cochromatographed with carrier cAMP. Similar results were obtained when α-[^{32}P]ATP was used as substrate for the enzyme.

Assay for cAMP. An adaptation of the method of Gilman (1970) was used. The packed yeast cells (0.1–0.2 g wet wt) were suspended in 2 ml of 10% trichloroacetic acid, centrifuged at 5000*g* for 10 min, and reextracted with 1 ml of 10%

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[1] Abbreviations used are: cAMP, cyclic 3′,5′-adenosine monophosphate; P-enolpyruvate phosphoenolpyruvate; PEI-cellulose, polyethyleneimine cellulose.

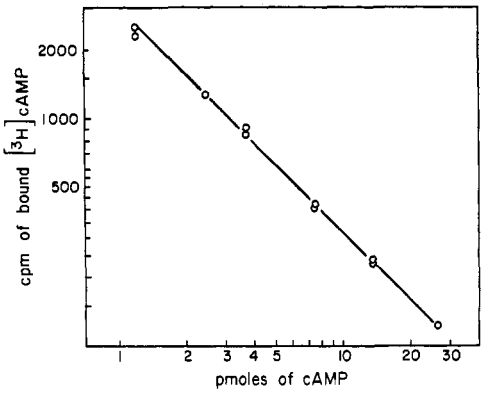

FIGURE 1: Standard curve for cAMP determination.

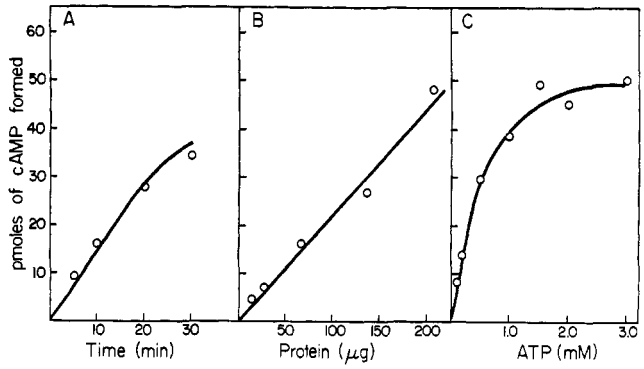

FIGURE 2: Formation of cAMP as a function of time (A), of membrane protein concentration (B), and of ATP (C). If not otherwise indicated, conditions for cAMP formation were the same as those described in the Experimental Section, except that 0.7 mM [^{3}H]ATP (A and B) and 275 μg of membrane protein (A and C) were used. In C, unlabeled ATP was added to 2.5 μCi of [^{3}H]ATP to obtain the desired final ATP concentration.

trichloroacetic acid and centrifuged again. To the combined supernatant fractions, one-tenth volume of 1 N HCl and two volumes of ether were added. The mixture was shaken in the cold for 10 min, the ether phase was discarded, the water phase was reextracted five times with ether, and then freeze-dried. The dried sample was dissolved in 0.1 ml of 100 mM phosphate buffer (pH 7.4) and aliquots were assayed for cAMP content by isotope dilution according to Gilman (1970). In a final volume of 50 μl, aliquots were mixed with 1.2 pmoles of [^{3}H]cAMP (12.7 Ci/mmole) in 0.1 M phosphate buffer (pH 7.4) and 10 mM $MgCl_2$. The binding reaction was then initiated by the addition of 13 μg of purified yeast cAMP binding protein (Sy and Richter, 1972); under these conditions, the amount of binding protein added will maximally bind 0.35 pmole of cAMP. The [^{3}H]cAMP concentration used was of saturating concentration. To complete equilibration, incubations were at 4° for 60 min. Samples were diluted with 2 ml of ice-cold buffer B (20 mM Tris-HCl (pH 7.6) and 10 mM $MgCl_2$) at the end of incubation, and the [^{3}H]cAMP protein complex was isolated by the Millipore filter technique. Intracellular cAMP concentrations were then calculated from standard curves similar to that shown in Figure 1. Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Materials. The yeast strains used were *S. fragilis* (10022) and *S. carlbergensis* (9080) from the American Type Culture Collection, and the wild-type strain *Saccharomyces cerevisiae* 18A and the "petite" mutant II-1-40 (the latter lacking mitochondrial DNA) which were obtained from Dr. J. Marmur. [^{3}H]cAMP and α-[^{32}P]ATP were obtained from Schwarz BioResearch, and PEI-cellulose thin-layer sheets from Brinkmann.

TABLE I: Distribution of Adenylyl Cyclase in the Yeast Homogenate.[a]

Fractions	Sp Act. (pmole/mg of Protein per 20 min)	Total Act. (%)
Homogenate (French press)	56	
1000*g* pellet	258	80
1000*g* supernatant fraction	22	20
Homogenate (spheroplast)	197	
1000*g* pellet	348	80
1000*g* supernatant fraction	42	20

[a] *S. fragilis* was grown in lactate medium and harvested at stationary phase as outlined in the Experimental Section of the preceding paper (Sy and Richter, 1972). The cells were either disrupted in a French press cell or transformed into spheroplasts and lysed osmotically (Davies and Elvin, 1964; Rozijn and Tonino, 1964). Homogenization was carried out in 20 mM phosphate buffer (pH 7.0), 5 mM $MgCl_2$, 0.25 mM EDTA, and 1 mM dithiothreitol. The assay for cyclase activity is described in the Experimental Section. Total activity of the combined pellet and supernatant fraction = 100%.

Results

Properties of a Membrane-Bound Adenylyl Cyclase from S. fragilis. More than 80% of the adenylyl cyclase activity in *S. fragilis* was present in the particulate fraction that sedimented by a 1000*g* spin and that consisted mainly of cell-wall and membrane fragments (Table I). When spheroplasts were used, adenylyl cyclase activity was again found in the membrane fraction, indicating that this enzyme is located in the plasma membrane rather than in the cell wall. Further washing of the particulate fraction did not solubilize the enzyme. Adenylyl cyclase activity was linear with time for at least 20 min (Figure 2A) and proportional to concentration up to 200 μg of protein (Figure 2B); formation of 3′,5′-cAMP was linear with increasing ATP concentrations up to 0.8 mM (Figure 2C). Optimal activity was found at pH 6.5. The cyclase was stimulated about 20% in the presence of 5 mM NaF, whereas 0.5 mM PP_i caused 20% inhibition. Neither pyruvate nor lactate had any effect on cyclase activity.

Effect of Growth Conditions. Using glucose (1.5%) or lactate as carbon source and vigorous aeration, cyclase activity increased three- to fourfold as the yeast entered the late-log or stationary phase (Figure 3A), followed, in the lactate culture, by an additional burst of activity at the later stationary phase. When cultured in a rotary shaker where aeration was relatively poor, a more striking difference between glucose- and

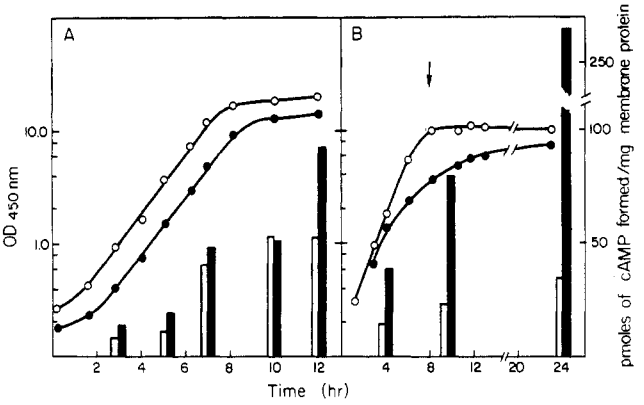

FIGURE 3: Growth curve and adenylyl cyclase activity of *S. fragilis*. (A) *S. fragilis* was grown overnight in a New Brunswick incubator rotary shaker at 37° and 250 rpm, in either 1.5% glucose or 2% lactate medium (Richter, 1971). Then 70 ml of lactate-grown cells (5.1 A_{450} units/ml) or 70 ml of glucose-grown cells (12.5 A_{450} units/ml) were transferred to 15-l. flasks of a New Brunswick fermentor containing 10 l. of either 2% lactate or 1.5% glucose medium. Growth conditions were as described in the Experimental Section of the preceding paper (Sy and Richter, 1972). After 3-, 5-, and 7-hr growth, 1 l. of cell culture was removed; after 10 and 12 hr, 0.5 l. was collected. The cell suspensions were cooled rapidly and the cells harvested in a continuous flow rotor. Cells were disrupted and assayed for cyclase activity as outlined in the Experimental Section. (B) In 2-l. flasks, 500 ml of 2% lactate or 1.5% glucose medium (Richter, 1971) were inocculated with 2 ml of a yeast suspension (10 A_{450} units/ml) adapted to the corresponding medium. Cells were grown in a New Brunswick rotary shaking incubator at 37° and 250 rpm. One flask of each medium was used for growth curve determination. At the indicated times, 500-ml cell cultures were rapidly chilled and harvested by centrifugation. Cyclase activity was determined as described in the Experimental Section. The arrow indicates the time when glucose was exhausted in the medium. Glucose and lactate concentrations of the medium were determined by the method of Barker (1957) and Ashwell (1957), respectively. The logarithmic growth curve was measured at 450 nm. (○) 1.5% glucose medium; (●) 2% lactate medium. Cyclase activity of the membrane fractions obtained from cells grown in 2% lactate medium (black column), or from cells grown in 1.5% glucose medium (white column).

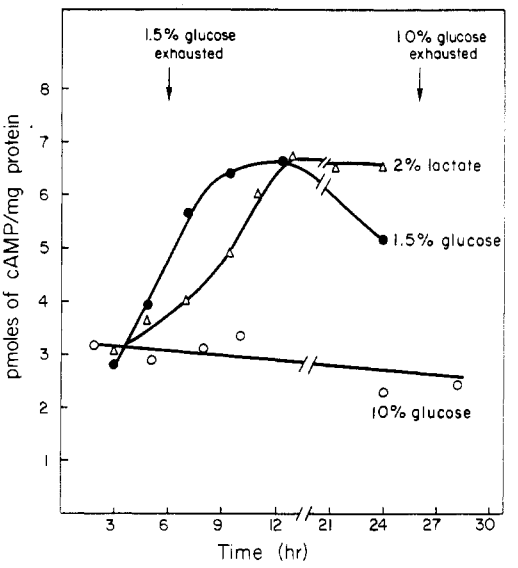

FIGURE 4: Intracellular cAMP content of *S. fragilis* grown in the presence of different carbon sources. Cells were cultured as outlined in the Experimental Section and in Figure 3B. At the times indicated, 5- to 20-ml aliquots were removed, the cells were rapidly chilled, centrifuged, treated with trichloroacetic acid, and assayed for cAMP content as described in the Experimental Section. Protein concentration of the trichloroacetic acid precipitate was determined by the method of Lowry *et al.* (1951). The arrows indicate the time when the glucose initially present in the culture was exhausted.

lactate-grown yeast was observed. Cyclase activity became seven- to eightfold higher in cells grown in lactate above those grown in glucose medium (1.5%) (Figure 3B). After incubation for 8 hr, glucose was exhausted whereas 50% of the lactate was still present after 24 hr. Cyclase activity was extremely low in cells grown under anaerobic conditions or in the presence of 10% glucose (not shown).

Since it was not possible to relate the adenylyl cyclase activity of *S. fragilis* to any specific regulatory process, it was thought that other yeast strains might be more suitable for this purpose. Surprisingly, both *S. cerevisiae* and *S. carlbergensis* show no, or very low, cyclase activity. One possible explanation was thought to be the technical difficulty of rupturing these cells with their more rigid cell wall. However, when spheroplast membranes of the same strains were prepared, they did not show better cyclase activity.

Intracellular cAMP Content in S. fragilis. S. fragilis was grown in medium with either lactate (2%) or glucose (1.5 or 10%) as carbon source. Cells were removed at different times and assayed for cAMP content as described in the Experimental Section. Figure 4 shows that the intracellular amount of cAMP increased considerably during growth when cells were cultured in lactate or low glucose medium (1.5%). This increase was almost linear for a period of 5–6 hr (Figure 4). Under both growth conditions the plateau of intracellular cAMP content was reached when the lactate- or glucose (1.5%)-grown yeast cells entered the stationary phase (after 12 and 9 hr, respectively). Comparison of the results of Figures 3 and 4 demonstrates that both the activity of the adenylyl cyclase and the intracellular cAMP concentration increased in parallel up to the stationary phase. Intracellular cAMP concentration was highest in lactate- or low glucose-grown cells at the early stationary phase, when adenylyl cyclase activity was also high. However, unlike the additional increase in adenylyl cyclase activity at the late stationary phase of lactate-grown cells, no such increase of intracellular cAMP was observed. Since we did not assay the extracellular cAMP, it remains to be seen whether this discrepancy could be attributed to secretion of the nucleotide into the medium (Peterkofsky and Gazdar, 1971).

Furthermore, Figure 4 shows that high glucose concentration (10%) in the medium seems to suppress the formation of cAMP. This appeared to parallel the observation that *E. coli* cells grown in high glucose medium accumulate less intracellular cAMP than cells grown in low glucose medium (Makman and Sutherland, 1965; Peterkofsky and Gazdar, 1971). When glucose was exhausted, the cAMP content inside the bacterial cells increased (Makman and Sutherland, 1965; Peterkofsky and Gazdar, 1971). With yeast, however, even after exhaustion of glucose (10%) the intracellular cAMP content does not rise. We do not know yet whether this may be related to the very low adenylyl cyclase activity observed under the same conditions.

Discussion

Recently, Van Wijk and Konijn (1970) reported an increase in intracellular cAMP in derepressed yeast cells (*S.*

carlbergensis). Since glucose is known for its repressive effect on inducible enzymes from yeast (Magasanik, 1961; Holzer, 1967; Linnane and Haslam, 1970), this result suggests that cAMP may have some regulatory function in the catabolic machinery. Although at present we cannot specify its function, from the data presented it is evident that cAMP, and even more so, adenylyl cyclase activity, are affected by the carbon source used and by the rate of aeration. Low aeration and lactate as carbon source in the culture appear to produce the highest cyclase activity in the stationary phase coincident with the highest intracellular cAMP content. Under anaerobic conditions or with high concentrations of glucose (10%) as substrate, almost no cyclase activity can be measured; under these conditions the intracellular cAMP concentration is relatively low. Whether the burst of cyclase activity at the late stationary phase of growth reflects a switch from actively growing to resting or even sporulating cells, and whether its product, the cAMP, functions in this process, remains to be elucidated. Since all yeast strains assayed here contained the highly specific cAMP binding protein, and at least two strains, *S. fragilis* and *S. carlbergensis*, (Van Wijk and Konijn, 1970) are known to contain intracellular cAMP, it seems likely that adenylyl cyclase may be found under appropriate conditions in yeast strains other than *S. fragilis*.

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