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Mechanism of Control of Adenylate Cyclase Activity in Yeast by Fermentable Sugars and Carbonyl Cyanide *m*-Chlorophenylhydrazone*

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The phosphorylation of fructose-1,6-bisphosphatase is preceded by a transient increase in the intracellular level of cyclic AMP which activates a cyclic AMP-dependent protein kinase (Pohlig, G., and Holzer, H. (1985) *J. Biol. Chem.* 260, 13818–13823). Possible mechanisms by which sugars or ionophores might activate adenylate cyclase and thereby lead to an increase in cyclic AMP concentrations were studied. Studies with permeabilized yeast cells demonstrated that neither sugar intermediates nor carbonyl cyanide *m*-chlorophenylhydrazone are able to increase adenylate cyclase activity. In the light of striking differences of the effects of fermentable sugars and of carbonyl cyanide *m*-chlorophenylhydrazone on parameters characterizing the membrane potential, it seems not reasonable that the activity of adenylate is under control of the membrane potential. Rapid quenching of 9-aminoacridine fluorescence after addition of fermentable sugars to starved yeast cells indicated an intracellular acidification. The ^{31}P NMR technique showed a fast drop of the intracellular pH from 6.9 to 6.55 or 6.4 immediately after addition of glucose or carbonyl cyanide *m*-chlorophenylhydrazone. The time course of the decrease of the cytosolic pH coincides with the transient increase of cyclic AMP concentration and the 50% inactivation of fructose-1,6-bisphosphatase under the conditions of the NMR experiments. Kinetic studies of adenylate cyclase activity showed an approximately 2-fold increase of activity when the pH was decreased from 7.0 to 6.5, which is the result of a decrease in the apparent K_m for ATP with no change in V_{max}. These studies suggest that activation of adenylate cyclase by decrease in the cytosolic pH starts a chain of events leading to accumulation of cyclic AMP and phosphorylation of fructose-1,6-bisphosphatase.

Addition of glucose or other fermentable sugars to glucose-derepressed yeast cells causes repression of the synthesis of enzymes participating in gluconeogenesis ("catabolite repression") (1) and moreover rapid inactivation of fructose-1,6-bisphosphatase and other key enzymes of gluconeogenesis ("catabolite inactivation") (2). In the case of fructose-1,6-bisphosphatase, a 50% inactivation resulting from a phosphorylation of the enzyme is observed within 1–3 min after addition of glucose (3–6). There is evidence that the covalent modification of fructose-1,6-bisphosphatase by phosphorylation marks the enzyme for selective proteolysis (5, 7) which is complete after 1–2 h (4, 8, 9). Studies of the mechanism of catabolite inactivation have revealed that the cAMP concentration increases about 5-fold within 30 s after addition of glucose (10, 11). This suggested that the phosphorylation of fructose-1,6-bisphosphatase might be dependent on cAMP (11, 12). In fact, *in vitro* experiments with purified fructose-1,6-bisphosphatase (13) and purified cAMP-dependent protein kinase from yeast (14, 15) or from beef heart (16) demonstrated phosphorylation of fructose-1,6-bisphosphatase accompanied by about 50% loss of activity of the enzyme. Moreover, it was shown that some of the kinetic properties of fructose-1,6-bisphosphatase (pH/activity profile, dependence of the reaction rate on the concentration of Mg^{2+} or Mn^{2+}) changed during *in vivo* phosphorylation after addition of glucose to starved yeast cells in the same way as during *in vitro* phosphorylation with the purified yeast enzymes (15). Similar to fermentable sugars, ionophores such as CCCP[1] or 2,4-dinitrophenol cause a transient increase of the concentration of cAMP (17) and phosphorylation of fructose-1,6-bisphosphatase (18) in glucose-derepressed yeast cells. This increase in cAMP concentration after addition of fermentable sugars or ionophores might be due to activation of adenylate cyclase and/or inhibition of cAMP phosphodiesterase. The present studies were designed to explore possible mechanisms by which glucose or CCCP might activate adenylate cyclase.

MATERIALS AND METHODS[2]

RESULTS

The rapid transient increase in cAMP level after addition of fermentable sugars or ionophores to starved intact yeast

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[1] The abbreviations used are: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; TPP^+, tetraphenylphosphonium; Pipes, 1,4-piperazinediethanesulfonic acid; GMP-PNP, guanyl-5′-yl (β,γ-imino)diphosphate.

[2] Portions of this paper (including "Materials and Methods," Table I, and Figs. 1, 3, and 5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-0196, cite the authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

cells suggested the possibility of activation of adenylate cyclase by glucose itself or by its metabolites. Accumulation of phosphorylated sugar derivatives after addition of glucose to starved yeast has been demonstrated previously (11, 33). It is shown in Fig. 1 that CCCP under the conditions used to demonstrate a transient increase of cAMP and inactivation of fructose-1,6-bisphosphatase also causes a transient accumulation of glucose 6-phosphate. Therefore, a direct demonstration of the effects of these sugars and of CCCP on adenylate cyclase activity in permeabilized cells was attempted. As shown in Table I, adenylate cyclase activity in yeast cells permeabilized by chitosan or toluene treatment was not significantly changed by addition of glucose or phosphorylation products of glucose. Also 2-dGlc and 6-dGlc as well as 2-dGlc-6-phosphate showed no significant effect. Of a variety of possible effectors tested a significant effect on adenylate cyclase activity was seen with inorganic phosphate. Addition of 5 mM P_i results in an apparently 50% increase in enzyme activity. This cannot explain the transient increase in cAMP, since it is known that the level of inorganic phosphate decreases rapidly after addition of glucose to starved yeast cells (34). Of interest is, however, the inhibition (approximately 40%) of adenylate cyclase activity by 1 mM AMP. It has been shown by von Herrath (35) in our laboratory that after addition of glucose to yeast under the conditions used in the experiments described here, the AMP concentration decreases in 1 min from 0.8 mM to about 0.2 mM. Therefore, a decrease of the AMP concentration after addition of glucose to starved yeast cells may activate adenylate cyclase by a deinhibition mechanism. The uncoupler CCCP, which, similar to glucose and other fermentable sugars, causes a transient increase in cAMP in intact cells (17) and also leads to a phosphorylation of fructose-1,6-bisphosphatase in intact cells (18) shows no significant direct effect on adenylate cyclase activity in permeabilized cells (Table I). Because 0.2 mM CCCP increases the AMP level 3-fold in starved yeast (data not shown) this ionophore in contrast to glucose cannot affect adenylate cyclase activity by release of AMP inhibition. In agreement with previous studies by Jaynes *et al.* (36) we found adenylate cyclase unaffected by 4 μM GMP-PNP, 1 mM KF, and 0.1 mM CCCP. This is in contrast to Casperson *et al.* (37) who described a stimulation of adenylate cyclase by GMP-PNP or GTP. Because the concentration of GTP does not increase immediately after addition of glucose or CCCP to starved yeast, a short term stimulation of adenylate cyclase by GTP might not be a reasonable explanation for the observed transient increase in cAMP.

As a next possibility for control of adenylate cyclase activity, dependence on the potential of the cell membrane was studied (*cf.* Ref. 38). As an indicator of the membrane potential the concentration of TPP^+ in the medium was measured with a TPP^+-selective electrode (27). Addition of fermentable sugars immediately and drastically decreases the extracellular TPP^+ concentration, *i.e.* increases the potential of the cell membrane (Fig. 2*A*). Ethanol, 2-dGlc, or 6-dGlc show no such effect (Fig. 2*B*). Measurements of the fluorescence of rhodamine, 6 G, as an indicator of the membrane potential (39) after addition of glucose produced completely parallel results (data not shown). The effects of the fermentable sugars, 2-dGlc, 6-dGlc, and ethanol, on the membrane potential parallel the effects on increase of the cAMP level (Ref. 11 and data not shown). However, 0.2 mM CCCP, which behaves like fermentable sugars with respect to the effect on increase of cAMP (upper part of Fig. 3), and on the decrease of catalytic activity of fructose-1,6-bisphosphatase as well as on phosphorylation of the 40-kDa subunits of fructose-1,6-bisphosphatase (lower part of Fig. 3) produces a different effect on membrane potential. As shown in Fig. 4, CCCP addition results in a slow increase of the extracellular $[^3H]TPP^+$, *i.e.* depolarization of the cell membrane. Since CCCP interferes with the assay of TPP^+ using the selective electrode, the concentration of ^3H-labeled TPP^+ in the medium was measured. After addition of glucose, the $[^3H]TPP^+$ showed a similar rapid decrease of the TPP^+ concentration in the medium (Fig. 4) in agreement with the data using the TPP^+-selective electrode (Fig. 2). Opposite effects of glucose and CCCP were also

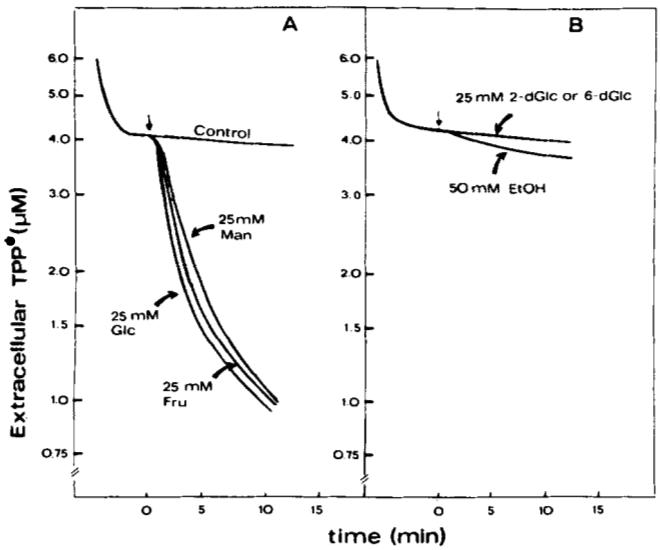

FIG. 2. **Effect of fermentable sugars (Glc, Man, Fru), nonfermentable sugars (2-dGlc, 6-dGlc), and ethanol on the extracellular concentration of TPP^+ (added as bromide) determined with a TPP^+ selective electrode.** Ethanol grown yeast suspensions (2.5% wet weight/v) were incubated in 25 mM Hepes/Tris, pH 7.0, at 30 °C (total volume 9 ml). The experiment was started by adding 9 μl of 6 mM TPP^+ (final concentration 6 μM). As indicated by the *vertical arrow*, different sugars or ethanol were added from 100 times concentrated stock solutions.

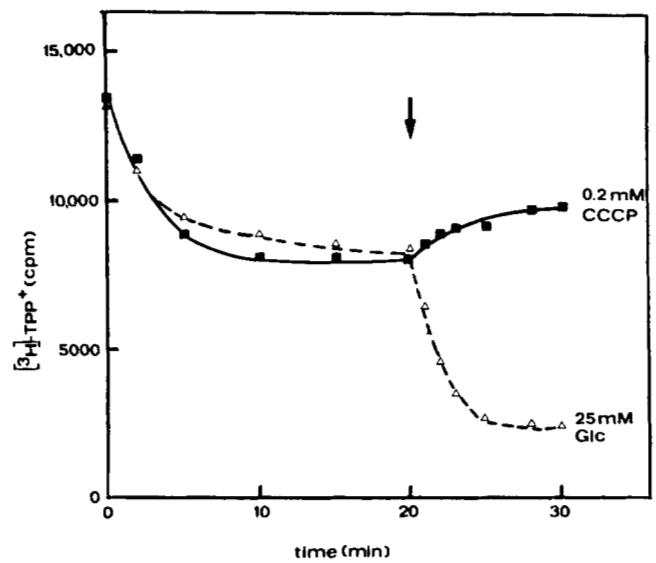

FIG. 4. **Effect of CCCP and glucose on extracellular TPP^+ concentration.** Ethanol grown, stationary yeast cells were incubated at 5% suspension (wet weight/v) in 50 mM Hepes/Tris, pH 7.0, at 30 °C. At zero time, 6 μM $[^3H]TPP^+$ was added. At the indicated times, 400-μl samples were withdrawn, centrifuged for 10 s, and 200 μl of supernatant counted. At 20 min, CCCP dissolved in methanol (final concentration 0.2 mM) or glucose (final concentrations 25 mM) were added.

obtained studying changes in the concentration of K^+ in the medium. As shown in Fig. 5, glucose causes a rapid decrease of the extracellular K^+ concentration, analogous to the decrease of the TPP^+ concentration shown in Figs. 2 and 4. In contrast, 0.2 mM CCCP, a concentration which increases cAMP, causes not a decrease but a slow increase in the extracellular K^+ concentration parallel to the slow release of [^{3}H]TPP^+ shown in Fig. 4. When CCCP is added after K^+ has reached the low plateau, it rapidly reverses the K^+ decreasing effect of glucose (Fig. 5). Because of the striking differences between the effects of fermentable sugars and CCCP on parameters characterizing the membrane potential, a control of adenylate cyclase activity by the membrane potential as an explanation for the mechanism of increase of cAMP initiated by fermentable sugars as well as by CCCP appears unlikely. The idea of control of adenylate cyclase activity by the intracellular pH was raised by results of Nicolay *et al.* (32). These authors used NMR spectra of inorganic phosphate for measurement of the cytosolic pH value of the yeast *Zygosaccharomyces bailii*. After addition of glucose to the starved yeast cells, a transient decrease of about 0.2 pH units was observed. Similar results have been reported by Den Hollander *et al.* (40) for *Saccharomyces cerevisiae*.

To check if under the conditions used in the present work addition of fermentable sugars also causes a decrease in the intracellular pH, changes of the fluorescence of 9-aminoacridine as an indicator of the intracellular pH (29, 30) were measured. As shown in Fig. 6, addition of fermentable sugars to starved yeast cells under the conditions where cAMP increases and fructose-1,6-bisphosphatase is phosphorylated causes distinct changes in fluorescence. 2-dGlc or 6-dGlc show no effects (CCCP cannot be studied because of heavy interference with the fluorescence measurements). With the continuous recording of the fluorescence it could be shown that

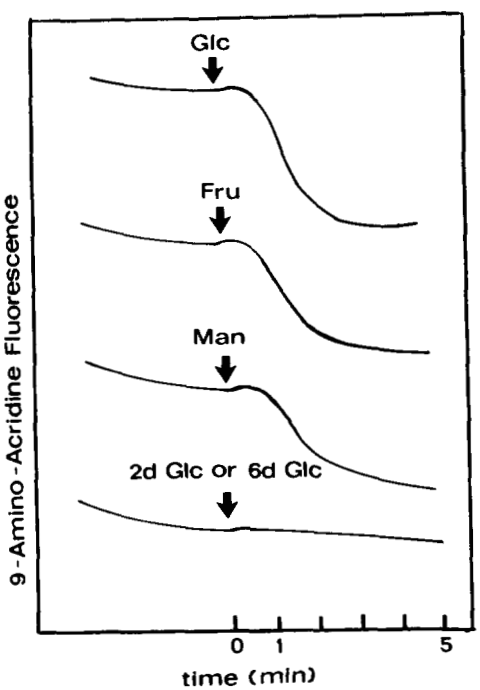

FIG. 6. **Effect of fermentable (Glc, Fru, Man) or nonfermentable (2-dGlc, 6-dGlc) sugars or ethanol on the intracellular pH.** Glucose grown yeast cells (30 mg wet weight) suspended in 3 ml of 50 mM Hepes/Tris, pH 7.0, were incubated at room temperature in the presence of 10 μM 9-aminoacridine for about 5 min until the decrease of the fluorescence was slow and linear. After this preincubation period, 25 mM fermentable or nonfermentable deoxy sugars were added from 2.5 M stock solutions.

the changes in intracellular pH are at least as rapid as the changes of the concentrations of glycolytic metabolites observed after addition of fermentable sugars (11, 33). The findings suggest that the activity of adenylate cyclase may be under control of the intracellular pH. There is controversy about whether changes of fluorescence of the dye actually measures a pH change or a membrane polarization effect. Therefore, a more direct measurement of changes of intracellular pH was done by applying the NMR technique used in the above-mentioned measurements of cytosolic pH values in *Z. bailii* (32). As shown in Table II, decrease of pH up to 0.35 units is observed after addition of glucose. In this experiment for technical reasons the concentration of yeast was raised from 2.5% or 5% wet weight/v used in the experiments shown in Figs. 1–6 to 40%. As depicted in Table II under exactly the same conditions of the NMR experiment with 40% yeast, inactivation of fructose-1,6-bisphosphatase and a transient increase of cAMP were observed as described for the experiments with 2.5% yeast suspensions. It is evident that under the nonphysiological conditions of 40% cell suspension the phenomenons being under study (transient increase of cAMP and inactivation of fructose-1,6-bisphosphatase) are observed similar to the 2.5–5% cell suspensions. With the NMR technique it was also possible to study the effect of CCCP on intracellular pH. Because of the high concentration of yeast (40%) necessary for the short term NMR experiments, instead of 0.2 mM CCCP (*cf.* Figs. 1, 4, and 5) 2 mM CCCP (final concentration) were added to the starved yeast. A decrease of 0.5 pH units within 1 min after addition of CCCP was observed (*cf.* Table II). Controls with addition of the solubilizer for CCCP (dimethyl sulfoxide) exhibited no change in the cytosolic pH. The effects of CCCP on fructose-1,6-bisphosphatase activity and cAMP concentration are shown in Table II. The results summarized in Table II demonstrate clearly that both CCCP and glucose decrease the cytosolic pH.

Activity of adenylate cyclase in permeabilized yeast cells at different pH values of the suspension medium was tested. It is shown in Fig. 7 that at pH 7, *i.e.* the cytosolic pH of starved yeast cells (32), a low activity of adenylate cyclase is observed. With both methods of permeabilization, chitosan or toluene treatment, a decrease of the pH below 7.0 causes distinct increases in the adenylate cyclase activity. Measurements of

TABLE II

Time course of the levels of fructose-1,6-bisphosphatase activity, pH in the cytosol, and cAMP in a 40% (wet weight/v) yeast suspension after addition of 2 mM CCCP or 2% Glc.

Determination of fructose-1,6-bisphophatase activity and assays of cAMP were done in simultaneous parallel experiments outside the magnet.

	Addition	
	Glc	CCCP
Specific activity of fructose-1,6-bisphosphatase (%)[a]		
5 min after addition	53	49
Cytosolic pH		
Before addition	6.90	6.90
1 min after addition	6.71	6.40
2 min after addition	6.62	6.40
3 min after addition	6.55	6.40
cAMP (nmol/g wet weight)		
Before addition	0.65	0.62
40 s after addition	1.2	0.88
80 s after addition	0.78	1.17
5 min after addition	0.82	0.98

[a] The reference value is that before addition of glucose or CCCP.

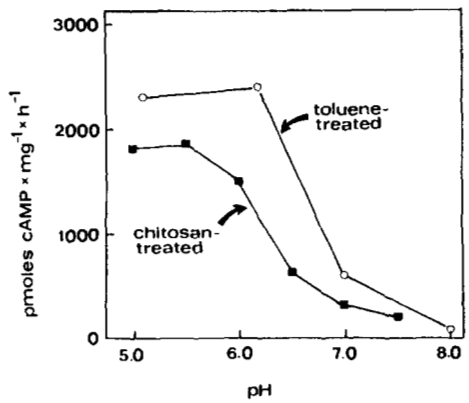

FIG. 7. **pH dependence of adenylate cyclase activity of glucose grown yeast cells permeabilized with toluene or chitosan.** The assay mixture contained 0.5 mM ATP, 0.4 mM cAMP, 5 mM $MnCl_2$ and was buffered in the case of toluene-treated cells with 0.1 M Mes/KOH, pH 5.1, 0.1 M Pipes/KOH, pH 6.2, 0.1 M Pipes/KOH, pH 7.0, or 0.1 M Hepes/KOH, pH 8.0, and in the case of chitosan-treated cells with 50 mM Mes adjusted with imidazole for the pH values 5.0–6.5 and 50 mM imidazole adjusted with Mes for the pH values 7.0 and 7.5. The reaction was started by addition of permeabilized yeast (0.2–0.5 mg of protein/ml).

TABLE III

ATP concentrations for half-maximal velocity (apparent K_m values) of adenylate cyclase activity as a function of the pH value

Glucose grown yeast cells were permeabilized with chitosan. The composition of assay mixtures was as described in the legend to Fig. 7 and the reaction was started by addition of permeabilized yeast (0.2 mg of protein/ml). The maximal velocity extrapolated from Lineweaver and Burk plots was at all pH values, 2400 pmol cAMP × h^{-1} × mg^{-1}.

pH	Concentration for half-maximal velocity
	mM
5.5	0.4
6.0	0.6
6.5	1.2
7.0	4.0
7.5	5.0

the dependence of adenylate cyclase activity of chitosan-permeabilized cells on the ATP concentration at different pH values showed that between pH 7.5 and 5.5 the apparent K_m values, but not the V_{max} values are dependent on the pH (Table III). The transient decrease of the cytosolic pH value after addition of glucose to starved yeast cells may therefore cause an activation of adenylate cyclase by increasing the affinity of the enzyme for ATP. This then, might account for the observed transient increase of cAMP.

DISCUSSION

In the present paper, evidence is accumulated in support of the hypothesis that a fast decrease of the intracellular pH after addition of glucose or of CCCP was responsible for *in vivo* activation of the adenylate cyclase. A similar hypothesis was mentioned by Caspani *et al.* (41) and by Valle *et al.* (42) for regulation by pH of the glucose induced, cAMP mediated activation of trehalase in *S. cerevisiae*. The NMR experiments summarized in Table II demonstrated definitively a decrease of the cytosolic pH after addition of fermentable sugars. Relatively high concentrations of CCCP are necessary to induce a cAMP increase, in contrast to low CCCP concentrations for uncoupling. The high CCCP concentrations increase the vacuolar pH to the level of the cytosolic pH, *i.e.* they destroy the compartmentation between vacuoles and cytosol

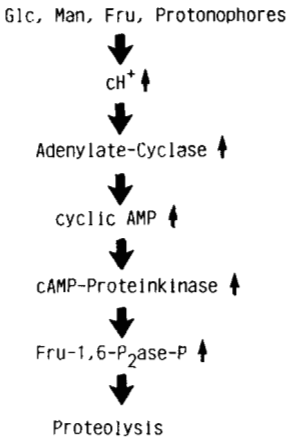

FIG. 8. **Proposed sequence of events after addition of fermentable sugars or CCCP to starved yeast.**

(data not shown). This is evidence that CCCP when stimulating adenylate cyclase acts as a "protonophore."

To investigate the pH dependence of adenylate cyclase activity we used permeabilized yeast cells for closer physiological conditions compared to membrane preparations obtained from broken cells. Fig. 7 demonstrates the pH dependence of toluene- and chitosan-treated yeast cells at 0.5 mM ATP. Within the range of the physiological pH a considerable increase of the adenylate cyclase activity from pH 7 to 6 is observed. As calculated from the Lineweaver-Burk plot for chitosan-treated cells (data not shown), V_{max} was unaffected between pH 5.5 and 7.5, whereas the ATP concentration for 50% activity ("apparent K_m") showed a minimum of 0.4 mM ATP at pH 5.5 and increased to 5 mM at pH 7.5. These findings are quite similar to the experimental values for toluene/ethanol-permeabilized cells reported by Varimo and Londesborough (43). Detailed pH kinetic studies of glucose-6-phosphatase, a membrane-bound enzyme intensively investigated in rat liver, revealed a very similar situation as with yeast adenylate cyclase, *i.e.* a marked dependence of the K_m value but no modulation of V_{max} as a consequence of changes in the pH of the assay mixture (44).

At first sight protons as effectors in metabolic regulation seems improbable because most enzymes and many other catalysts in a variety of different metabolic pathways would respond at the same time to a change of the proton concentration. This would be in contrast to the pathway specific actions known for many regulatory effectors. Nevertheless, in recent years more and more control mechanisms affected by changes in the pH have been described (for a recent summary see Ref. 45). It may be that membrane-bound enzymes, such as liver glucose-6-phosphate phosphatase (44) or yeast adenylate cyclase are preferred candidates for regulation by proton concentration.

The regulation of adenylate cyclase activity by the proton concentration fills a gap in the chain of events leading from application of fermentable sugars or protonophores to proteolysis of fructose-1,6-bisphosphatase (Fig. 8). In this process, which is part of "catabolite inactivation" in yeast (2), a rapid phosphorylation of fructose-1,6-bisphosphatase (5, 6) preceded by a transient increase of cAMP (11) had been demonstrated previously in intact yeast cells. A cAMP- and fructose 2,6-bisphosphate-dependent phosphorylation of fructose-1,6-bisphosphatase has been demonstrated with purified enzymes from yeast (14, 15). Dependence of proteolysis of fructose-1,6-bisphosphatase on cAMP-dependent phosphorylation of the enzyme was demonstrated with adenylate cyclase-deficient mutants of *S. cerevisiae* (7). The question how a

rapid increase of cAMP is affected by application of fermentable sugars or protonophores to starved yeast may now be answered by the demonstration of a rapid decrease of the cytosolic pH which causes activation of adenylate cyclase. These studies do not explain the transiency of the increase of the cAMP level. It is possible that a change in the activity of cAMP phosphodiesterase also participates in the control of the level of cAMP.

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Mechanism of control of adenylate cyclase activity in yeast by fermentable sugars and carbonyl cyanide m-chlorophenylhydrazone

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Materials and Methods

For all experiments the diploid strain M1 of Saccharomyces cerevisiae, kindly provided by Prof. Linnane (Monash University, Clayton, Australia), was used. The yeast cells were cultivated on YEPD-medium (1% Bacto yeast extract, 2% Bactopeptone and 2% glucose) or Ethanol-medium (0.67 % Yeast Nitrogen Base w/o Amino acids, 0.5% ethanol and 50 mM Mes /KOH, pH 5.6) and harvested after 24 h or 48 h, respectively.

Enzyme assays and in vivo phosphorylation of fructose-1,6-bisphosphatase - Fructose-1,6-bisphosphatase was assayed spectrophotometrically with a modification (13) of the method described by Racker and Schröder (19). Adenylate cyclase activity in toluene-treated cells and chitosan-treated cells was measured according to Salomon et al. (20), a modification of the original method of Krishna et al. (21). Reaction rates at 30°C were linear for 20 to 30 min without an ATP-regenerating system, whereas without addition of 0.4 mM cAMP a significant lower activity was measured. For details concerning the termination of the enzyme reaction, separation of ^{32}P - labeled cAMP and recovery controls with ^{3}H cAMP, see Harwood and Peterkofsky (22). Protein was determined according to Bradford (23) using the Bio-Rad test solution. In vivo phosphorylation of fructose-1,6-bisphosphatase and immunoprecipitation of the crude extract was carried out as described by Müller and Holzer (5). The antiserum was kindly provided by Dr. T. Noda (13). Phosphorylation of fructose 1,6-bisphosphatase in the immunoprecipitates was measured by scanning the autoradiograms (Kodak Film XAR-5) of slab gels with a LKB 2202 ultroscan laser densitometer. Gel electrophoresis was performed in gradients of 10-20% polyacrylamide using the procedure of Laemmli (24) and proteins were stained with Coomassie Brilliant Blue R250.

Metabolites - Concentrations of glucose-6-phosphate, ATP and cAMP were determined after extraction with perchloric acid as previously described (11).

Cell permeabilization - Toluene treatment was expected to be a suitable method for measuring adenylate cyclase activity exhibiting characteristics closer to the in vivo condition than membrane preparations (22). The yeast suspension (1 g packed yeast cells suspended in 4 ml 0.1 M Pipes/KOH, pH 6.2) was shaken in the presence of 16 ml toluene for 5 min at 40°C as described by Murakami et al. (25). The cells were washed twice by centrifugation at 10,000 xg for 10 min and resuspended in the same buffer. They could be stored at 4°C for several days without significant loss of activity. For chitosan treatment, the procedure of Jaspers et al. (26) was slightly modified to obtain permeabilization at 0°C. 0.75 g packed yeast cells were suspended in 150 ml 25 mM Hepes/Tris pH 7.0 containing 50 mM KCl and stirred for 5 h in the presence of 7.5 mg chitosan. After sedimentation of the yeast cells the supernatant was aspirated. Adenylate cyclase showed an about 40% decrease in activity after two days during storage at 0°C. Only freshly permeabilized yeast cells were used for the described experiments.

Membrane potential and extracellular potassium - TPP^+ (27) and extracellular potassium (28) concentrations (as indicators of the membrane potential) were measured with ion selective electrodes. The Ag/AgCl/3 M KCl - half cell of a combination pH electrode EA 125 (Metrohm, Herisau, F.R.G.) was used as reference electrode. For the measurement of potassium concentration, the half-cell contained 3 M NaCl instead of 3 M KCl. The electrode potentials were measured with pH meter E510 (Metrohm, Herisau, F.R.G.) and recorded by a multi-pen recorder R103 (Rikadenki, Freiburg, F.R.G.).

Intracellular pH - A simple fluorescence assay using 9-aminoacridine (29,30) as a pH indicator was used. Intracellular acidification causes an accumulation of the dye inside the cell, because protonation of the dye decreases its ability to pass through the plasma membrane. Fluorescence quenching, observed when 9-aminoacridine is taken up by the yeast cells, could therefore be interpreted as lowering of the intracellular pH. Fluorescence was measured with a spectrofluorometer RRS 1000 (Schoeffel, Westwood, NJ, USA) (excitation wavelength 397 nm, emission wavelength 454 nm) and recorded with an XY flat bed recorder PM 8125 (Philips, Eindhoven, Netherlands).

^{31}P-NMR - After harvesting, the cells were washed twice in ice-cold resuspension medium containing 0.1 M Hepes/Tris, pH 7.0, and resuspended in the same medium. The cell pellet volume was usually 30 - 40% of the total sample volume. ^{31}P NMR spectra were obtained at 145.8 MHz using a Bruker HX-360 spectrometer operating in the Fourier-transform mode. Accumulation was carried out employing 60 pulses and a 0.34 s repetition time. Routinely, time profiles were obtained by sequentially storing on disk free induction decays, each consisting of 100 to 250 scans. Glycerophosphoryl choline at 0.49 ppm relative to 85% orthophosphoric acid was used as an internal chemical shift marker. Unless stated otherwise, NMR experiments were carried out at 22 ± 1°C. Samples consisted of 3 ml of the yeast suspension in 10 mm tubes. Aerobic conditions in the NMR tube were maintained by bubbling pure O_2 gas through the dense cell suspension. In all experiments, gas was bubbled through a glass capillary (31) at a rate of 30 - 40 ml/min. Prior to each experiment, 50 µl antifoam was added to the cell suspension. Substrates were injected directly into the NMR tube inside the magnet. This was achieved by introducing 150 µl 50% glucose solution into a bypass of the silicone tubing used for the gas supply. Subsequently, the gas flow was directed through the bypass, thus pushing the substrate solution into the yeast suspension (31). CCCP (12 µl, 0.5 M solution in dimethylsulfoxide) was added to the NMR tubes outside the magnet.

Intracellular pH - The pH of the cytosol and vacuole was determined by ^{31}P NMR from the chemical shifts of inorganic phosphate in the specific compartments as described elsewhere (32).

Table I
Adenylate cyclase activity in cells of the yeast Saccharomyces cerevisiae M1 permeabilized by chitosan-treatment or toluene-treatment.

Addition	Chitosan-treated		Toluene-treated	
	Concentration of addition (mM)	Activity (%)	Concentration of addition (mM)	Activity (%)
None	—	100[1]	—	100[2]
Glc	5	88	3	90
2-dGlc	5	85	3	82
6-dGlc	5	95	n.t.	n.t.
Glc-6-P	5	97	3	99
2-dGlc-6-P	5	110	3	127
Glc-1-P	n.t.	n.t.	1	95
Fru-6-P	5	97	3	113
Glc-1,6-P_2	n.t.	n.t.	1	93
Fru-1,6-P_2	5	110	3	114
Fru-2,6-P_2	0.005	109	0.1	114
P_i	5	142	n.t.	n.t.
P_i	15	164	n.t.	n.t.
AMP	0.1	95	n.t.	n.t.
AMP	1	63	n.t.	n.t.
GMPPNP	n.t.	n.t.	0.004	100
KF	n.t.	n.t.	1	95
CCCP	0.1	81	n.t.	n.t.

Table I
The yeast cells were cultivated on YEPD-medium. Mixtures for assay of adenylate cyclase contained in the case of chitosan-pretreatment (final concentration): 50 mM Imidazole/Mes pH 7.0, 1.0 mM ATP, 5 mM $MnCl_2$, 0.4 mM cAMP and cells (0.2 mg/ml protein) and in the case of toluene-pretreatment: 100 mM Mes/KOH pH 5.5, 0.5 mM ATP, 0.5 mM $MnCl_2$, 0.4 mM cAMP and cells (0.6 mg/ml protein). Protein content of cells was measured in a French pressed suspension.
1) actual value: 670 pmoles/h/mg 2) actual value: 1100 pmoles/h/mg
n.t.: not tested

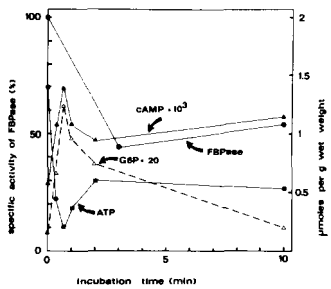

Fig. 1
Changes in specific activity of fructose-1,6-bisphosphatase (FBPase) and concentrations of cAMP, ATP and glucose-6-phosphate (G6P) after addition of CCCP (final concentration 0.1 mM) to a 2.5% (wet weight per volume) yeast suspension in 10 mM sodium maleate pH 6.0 at 30°C.

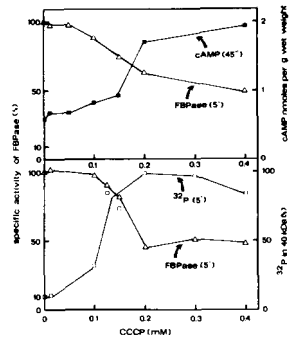

Fig. 3
Effect of increasing concentrations of CCCP on the activity of and ^{32}P incorporation into fructose-1,6-bisphosphatase (FBPase) and on levels of cAMP. The yeast cells cultivated on YEPD-medium were incubated at a density of 5% wet weight/vol in 50 mM Hepes/Tris, pH 7.0 at 30°C. CCCP dissolved in methanol was added after 5 min preincubation. Samples were withdrawn for determination of cAMP 45 sec after CCCP and for determination of enzyme activity or enzyme phosphorylation 5 min after CCCP.

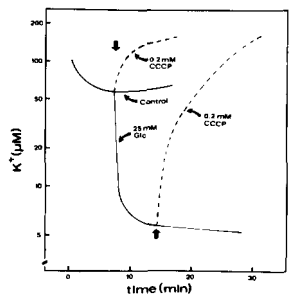

Fig. 5
Effect of glucose and CCCP on the extracellular K^+ concentration of glucose grown, stationary yeast. Washed yeast cells (25 mg wet weight/ml) were stored overnight at 0°C in 50 mM Hepes/Tris, pH 7.0, centrifuged the next day and resuspended prior to the experiment in the same buffer. As indicated by vertical arrow 25 mM glucose from a 2.5 M stock solution or 0.2 mM CCCP (dotted lines) from a 100 mM stock solution in methanol were added.