Effect of carbon source on the accumulation of cytochrome *P*-450 in the yeast *Saccharomyces cerevisiae*

Sirpa O. KÄRENLAMPI,* Eine MARIN* and Osmo O. P. HÄNNINEN†

Departments of *Biochemistry and †Physiology, University of Kuopio, P.O. Box 138, SF-70101 Kuopio 10, Finland

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The appearance of cytochrome *P*-450 in the yeast *Saccharomyces cerevisiae* depended on the substrate supporting growth. Cytochrome *P*-450 was apparent in yeast cells grown on a strongly fermentable sugar such as D-glucose, D-fructose or sucrose. When yeast was grown on D-galactose, D-mannose or maltose, where fermentation and respiration occurred concomitantly, cytochrome *P*-450 was also formed. The cytochrome *P*-450 concentration was maximal at the beginning of the stationary phase of the culture. Thereafter the concentration decreased, reaching zero at a late-stationary phase. When the yeast was grown on a medium that contained lactose or pentoses (L-arabinose, L-rhamnose, D-ribose and D-xylose), cytochrome *P*-450 did not occur. When a non-fermentable energy source (glycerol, lactate or ethanol) was used, no cytochrome *P*-450 was detectable. Transfer of cells from D-glucose medium to ethanol medium caused a slow disappearance of cytochrome *P*-450, although the amount of the haemoprotein still continued to increase in the control cultures. Cytochrome *P*-450 appeared thus to accumulate in conditions where the rate of growth was fast and fermentation occurred. Occurrence of this haemoprotein is not necessarily linked, however, with the repression of mitochondrial haemoprotein synthesis.

The physiological state of the cells of the yeast *Saccharomyces cerevisiae* depends greatly on environmental conditions. This yeast is a facultative anaerobe whose metabolism differs depending on whether the growth occurs in the presence or absence of O₂. The amount of aeration seems to play an important role also in the appearance of cytochrome *P*-450 in this yeast (Lindenmayer & Smith, 1964; Ishidate *et al.*, 1969*a,b*). In addition to O₂, the carbon and energy source can be used to modify the type of metabolism in yeast. Most studies of cytochrome *P*-450 in *Saccharomyces cerevisiae* have been done with a glucose-containing medium. Wiseman *et al.* (1975*a*) showed that the concentration of cytochrome *P*-450 was independent of glucose concentrations above 1% in yeast grown aerobically. Wiseman & Lim (1975) observed no production of cytochrome *P*-450 on 0.5% glucose. Callen & Philpot (1977) reported a tenfold greater accumulation of cytochrome *P*-450 on glucose compared with galactose as the carbon source.

The aim of the present study was to clarify the regulation of cytochrome *P*-450 concentrations in *S. cerevisiae* by using different types of carbon and energy sources. For this purpose the yeast was

grown on various fermentable substrates that repress respiratory-enzyme synthesis, on substrates that permit both fermentation and respiration, and on substrates that support only respiration.

Experimental

Organism

The yeast *Saccharomyces cerevisiae* Hansen, strain no. 240, was obtained from Mrs. Barbara Kirsop, The National Collection of Yeast Cultures, Brewing Industry Research Foundation, Nutfield, Surrey, U.K., whom we gratefully acknowledge. The yeast was maintained on agar slopes containing 20 g of D-(+)-glucose/litre.

Media

The growth medium contained (in 1 litre of deionized water): 10 g of yeast extract, 20 g of peptone, 5 g of NaCl and the main carbon and energy source. Carbon sources were sterilized separately and used at different final concentrations. The pH values of media at the beginning of growth were adjusted, if necessary, to 4.7–5.5. The medium without added carbon source contained 0.14 g of

glucose/litre. D-Galactose was found to contain 2.5mg of glucose/g. The other sugars used (D-fructose, D-mannose, L-arabinose, L-rhamnose, D-ribose, D-xylose, sucrose and lactose) contained no, or only traces of, glucose.

Culture conditions

The yeast was grown as batch cultures. New slopes, which contained the same energy sources (20g/litre) used in experimental cultures, were cultured for 2 days at 30°C. A whole slope was used to inoculate 1 litre of prewarmed medium held in cotton-plugged 3-litre Erlenmeyer flasks. These experimental cultures were incubated at 30°C in a water bath fitted with a thermostat (Heto, Birkerød, Denmark). Aeration, when used, was carried out with an aquarium pump, through a 1-ml pipette.

Harvest shift procedure

Half of the cells grown on D-glucose (or ethanol) medium to the exponential-growth phase were harvested by centrifugation (2500g, 5 min, Sorvall GLC-2 centrifuge) and resuspended into ethanol (or D-glucose) medium. The other half were kept as a control culture.

Measurement of culture growth

Culture cell density was measured photometrically at 600nm with a Coleman 54 spectrophotometer (Perkin-Elmer). A relationship similar to the Beer's Law of Absorption existed where absorbance readings were low. At higher densities there was an increasingly greater underestimation of absorbances when compared with the values obtained after dilution of the cell suspensions with fresh medium (see also, e.g., Lawrence & Maier, 1977). At $A_{0.5}$ the underestimate was about 15%, and at $A_{1.0}$, about 50%. The values presented in the Figures are uncorrected. $A_{0.5}$ corresponded to 10.2mg wet wt./ml of growth culture. Wet weight was 5.4 times greater than dry weight.

Assay of cytochromes P-450 and 'P-420' from whole cells

Samples corresponding to about 0.6g wet wt. of yeast were removed aseptically from cultures and harvested by centrifugation at 2500g for 5 min. The cells were washed with cold deionized water and stored frozen (-20°C) until analysed. The measurement of cytochrome P-450 was carried out principally as described by Omura & Sato (1964). For the assay the samples were thawed and suspended in 6ml of cold 0.1M-potassium phosphate buffer, pH 7.4. A few grains of sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) were added to complete the reduction of cytochromes P-450 and 'P-420', and the suspension was divided between two cuvettes. CO was bubbled through the sample cuvette. The recordings of the

CO difference spectra were made at room temperature in an automatically scanning Cary model 118 (Varian) spectrophotometer. The heights of the peaks were measured at the wavelengths of maximal absorbances near 450 and 420nm. The approximate content of cytochrome P-450 was calculated by assuming that the difference of millimolar absorptivity of the haemoprotein between about 450 and 500nm is $92 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$ (Yoshida *et al.*, 1977). The results are expressed as nmol of cytochrome P-450/mg of protein (the s.e.m. was 10%; $n = 10$). The height of the peak at about 420nm was measured as the difference between the maximum at 420nm and the minimum between the bands at 450 and 420nm (cf. Cartledge & Lloyd, 1972). This calculation method differs from that described by Omura & Sato (1964). The results are expressed as absorbance differences/protein concentration (1 mg/ml).

Breakage of cells

A sample of cells (0.6g wet wt.) was suspended in 3 ml of 0.1M-potassium phosphate buffer, pH 7.4. It was shaken for 1min in a Braun MSK cell homogenizer (B. Braun Melsungen G.m.b.H., Melsungen, Germany) at a frequency of 4000 oscillations/min with 3g of glass beads (0.45–0.5mm diam.), cooling being carried out with liquid CO_2 . Buffer (3 ml, containing 7.5mg of sodium cholate) was added, and after 20min the suspension was centrifuged for 20min at 20000g (see Callen & Philpot, 1977).

Protein assay

This was measured from whole yeast cells by a modified biuret method (Stewart, 1975) in which the cells were first heated in a boiling-water bath for 15min in alkaline solution. Bovine serum albumin was used as a standard. The s.e.m. for the determination was 0.7% ($n = 13$).

Glucose and ethanol

Glucose was determined by the hexokinase/glucose 6-phosphate dehydrogenase method of Schmidt (1961). Ethanol was determined with alcohol dehydrogenase by the method of Bücher & Redetzki (1951).

Reagents

Bacto yeast extract and Bacto agar were purchased from Difco Laboratories, Detroit, MI, U.S.A. Bacteriological peptone was from Oxoid Ltd., London S.E.1. U.K. Malt extract was from Oy Rohto A.B., Tammisaari, Finland. L-(+)-Rhamnose ('puriss., for bacteriology') was from Fluka A.G., Buchs, Switzerland. Sucrose and lactic acid (AnalaR grade) were obtained from BDH, Poole, Dorset, U.K. Ethanol (99.5%) was from Oy Alko Ab,

Helsinki, Finland. Bovine serum albumin was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. NaOH (puriss.) was obtained from Elektrokemiska Aktiebolaget, Bohus Surte 1, Sweden. From E. Merck, A.G., Darmstadt, Germany were: D-(+)-glucose ('for biochemistry and microbiology'), D-(+)-galactose (puriss.), D-(–)-fructose ('for bacteriology'), L-(+)-arabinose ('for bacteriology'), D-(+)-mannose ('for biochemistry and microbiology'), maltose, D-(+)-xylose, lactose ('for microbiology'), D-(–)-ribose ('for biochemistry') and sodium dithionite and other chemicals (p.a. grade). Gluco-quant and blood-alcohol reagent kits were from Boehringer Mannheim G.m.b.H., Mannheim, Germany. CO was prepared from formic acid by dropwise addition of concentrated H₂SO₄. All aqueous reagents were made up with deionized water.

Results

Cultures in the presence of sugars

Cytochrome P-450 was apparent in whole yeast cells grown on a medium supplemented with a fast-fermenting main carbon and energy source, such as D-glucose, D-fructose or sucrose, or a carbon source supporting fermentation and respiration simultaneously, such as D-galactose, D-mannose or maltose (Table 1). There were some small differences in the maximal contents of cytochrome P-450 between these different carbon sources. The cytochrome P-450 concentration during the growth was

maximal in the beginning of stationary phase of growth (Fig. 1). When grown on a medium without added sugar, when the actual amount of D-glucose was 0.14 g/litre (see the Experimental section), no cytochrome P-450 was evident. The addition of 7.5 g of D-mannose/litre to the medium caused the appearance of cytochrome P-450. All the aforementioned sugars supported the synthesis of cytochrome P-450 when supplied at concentrations of 10 g/litre and higher.

A mild aeration generally lowered the amount of cytochrome P-450 in yeast when cultivated on D-glucose-, D-galactose- or D-mannose-containing medium, as compared with cultures without aeration (Fig. 2). This amount of aeration clearly increased the total mass of yeast formed from a certain amount of sugar.

Growth of yeast was fast on media containing these six sugars (D-glucose, D-fructose, sucrose, D-galactose, D-mannose and maltose). On 100 g of D-glucose/litre, exponential growth occurred before D-glucose utilization decreased the substrate concentration to about 14 g/litre, in agreement with the results of Cartledge & Lloyd (1972). The concentration of energy substrate decreased most rapidly during the late-exponential growth phase and the D-glucose concentration fell to zero when the cells started the stationary phase.

A peak near 420 nm in the reduced CO difference spectra, (cytochrome 'P-420') was found in all cultures (Fig. 1). It was low in the exponential growth phase. Normally the height of the peak began to increase strongly when the cells entered the stationary phase. The amount of 'cytochrome P-420' became maximal later than cytochrome P-450 in the stationary growth phase. In aerated cultures, the

Table 1. Production of cytochrome P-450 in yeast during growth on different carbon sources

Yeast was grown on a medium that contained different main carbon and energy sources at a concentration of 50 g/litre (except sucrose, which was used at a concentration of 100 g/litre). The results quoted are maximal values observed during growth and are the mean results of assays from independent cultures (the numbers of cultures are given in parentheses).

Carbon source	[Cytochrome P-450 _{max.}] (nmol/mg of protein)
D-Mannose	0.050 (2)
D-Fructose	0.049 (2)
D-Glucose	0.044 (6)
Sucrose	0.043 (1)
D-Galactose	0.038 (4)
Maltose	0.032 (1)
Lactose	0
Pentoses (L-arabinose, L-rhamnose, D-ribose and D-xylose)	0
Ethanol	0
Glycerol	0
Lactate	0

Fig. 1. Dependence of the amount of cytochromes P-450 and 'P-420' on the growth stage in yeast

The yeast was grown without aeration on a medium that contained 50 g of D-galactose/litre. O, Growth (*A*₆₀₀); Δ, cytochrome P-450 (nmol/mg of protein); □, 'cytochrome P-420' [*A*/protein concentration (1 mg/ml)].

Fig. 2. Effect of aeration on the amount of cytochrome P-450 during growth of yeast

The yeast was grown on mannose (50 g/litre)-containing medium, with or without aeration. ○, Growth without aeration (A_{600}); ●, growth with aeration; △, cytochrome P-450 from cells grown without aeration (nmol/mg of protein); ▲, cytochrome P-450 from cells grown with aeration.

maximum 'cytochrome P-420' formation came earlier but the amount formed was less. Aeration during growth also caused the trough between cytochrome P-450 and 'cytochrome P-420' to move to higher wavelengths (Fig. 3). The maximal amount of 'cytochrome P-420' was higher in galactose than in glucose or fructose grown cells. Also the amount of 'cytochrome P-420' at the time cytochrome P-450 reached a maximum was highest on galactose cultivations. 'Cytochrome P-420' accumulated later on fructose than on galactose.

On media containing other sugars [namely lactose and pentoses (L-arabinose, L-rhamnose, D-ribose, D-xylose)], growth of the yeast was very poor. The growth rate was of the same order of magnitude as that on a medium without added sugar. Cytochrome P-450 was not apparent. 'Cytochrome P-420' was maximal in the exponential growth phase on lactose.

Shift experiments

The rapid decrease of the concentration of energy

Fig. 3. Effect of aeration on the reduced CO difference spectra of yeast

Yeast was grown on mannose (50 g/litre) containing medium with or without aeration. The spectra were obtained from cells during growth when the amounts of cytochrome P-450 were maximal. —, Growth without aeration; ----, growth during aeration.

substrate (e.g. D-glucose) during growth was accompanied by a rapid increase in the concentration of ethanol. No ethanol was formed after the cells started the stationary phase. The yeast strain used accumulated about 40 g of ethanol/litre from media containing 100 g of D-glucose, D-galactose or sucrose.

Aerobic growth on glucose produces a diauxic growth (Polakis & Bartley, 1965), which is characterized by fast fermentation of glucose in the first exponential growth phase, followed by the oxidation of the accumulated ethanol during the second exponential phase. In the present experiments, these two stages were separated from each other. When grown on glucose without aeration, only the first exponential phase could be distinguished. During

Fig. 4. Cytochrome *P*-450 in yeast cells after shift from ethanol to glucose medium or vice versa

(a) The cells were cultured on ethanol (20 g/litre) containing medium and half of the cells were transferred (arrow) to glucose (50 g/litre) medium; (b) The cells were cultured on glucose (50 g/litre) containing medium and half of the cells were transferred (arrow) to ethanol (20 g/litre) containing glucose-depleted medium. O, Growth on ethanol (A_{600}); ●, growth on glucose; △, cytochrome *P*-450 from cells grown on ethanol (nmol/mg protein); ▲, cytochrome *P*-450 from cells grown on glucose.

this phase the pH fell from 5.4 to 4.6 as acetate accumulated. CO_2 formation was rapid. The second phase, in which yeast cells grow by oxidizing the products of sugar fermentation was not seen, and ethanol was not consumed. In the present experiments the second phase was demonstrated by transferring cells growing semi-anaerobically on glucose in the exponential phase to medium containing 20 g of ethanol, which had been aerated (Fig. 4). The growth on ethanol was slower than that on glucose. Transfer of the cells, previously grown on medium containing 50 g of glucose/litre, to a de-repression medium (ethanol) resulted in the disappearance of all preformed cytochrome *P*-450 within 50 h. When the yeast cells were shifted from glucose-depleted ethanol medium to glucose-containing medium, cytochrome *P*-450 began to accumulate after a short lag period and reached a maximum in about 30 h. The shift from glucose to ethanol caused the 'cytochrome *P*-420' to increase more rapidly than in the control culture. On the other hand, a shift from ethanol to glucose caused a very marked decrease in 'cytochrome *P*-420'. Within 9 h the amount of 'cytochrome *P*-420' decreased to one-fourth of the amount at the moment of the shift. It reached a minimum when cytochrome *P*-450 reached a maximum.

Effect of non-fermentable carbon sources

Cytochrome *P*-450 was not detected in whole

yeast cells when the cells were grown on a non-fermentable carbon-and-energy source like glycerol, lactate or ethanol. Breakage of the cells and cytochrome *P*-450 assay of sodium cholate-treated 20000g supernatant also did not give any clear evidence of the presence of cytochrome *P*-450. Lactate was consumed very slowly by the cells. On medium containing glycerol and ethanol, the rate of growth of cells was much faster, but still lower than that on energy sources of glucose type. 'Cytochrome *P*-420' was found in the cells with all these energy sources, and was highest in the exponential growth phase.

Discussion

Pasteur and Crabtree effects have been under intensive study in yeast. Metabolic events in the shift from fermentation to respiration have been screened in many ways. On the basis of the effects of aeration and glucose, it has been suggested that the accumulation of cytochrome *P*-450 in yeast requires a mitochondrial repression (Wiseman *et al.*, 1975a,b) and that its production is controlled by the intracellular concentration of 3',5'-cyclic AMP (Wiseman, *et al.*, 1975a, 1978). This suggestion is supported by the observation that the amount of cytochrome *P*-450 is so low in galactose-grown yeast that it cannot be detected in whole cells (Callen & Philpot, 1977).

One way to clarify further the regulation of cytochrome *P*-450 in yeast is to use different types of carbon sources. When *S. cerevisiae* is grown exponentially on glucose or fructose as carbon-plus-energy source, and in the presence of air, the glucose degradation proceeds mainly via aerobic fermentation (DeDeken, 1966). Galactose, mannose and maltose inhibit respiration less, and the degradation of these sugars may proceed through fermentation and respiration simultaneously. The present experiments showed that cytochrome *P*-450 accumulated when the cells were grown on sugars whose degradation proceeds more or less via aerobic fermentation. Cytochrome *P*-450 accumulated although degradation of the sugar proceeded simultaneously via respiration. This is also supported by the fact that, during the 'fermentative phase of de-repression' (Perlman & Mahler, 1974), cytochrome *P*-450 was still formed, although de-repression of a variety of intra- and extra-mitochondrial activities can be detected. Cytochrome *P*-450 did not, however, appear when the substrate was non-fermentable. Also the second phase, 'oxidative or respiratory phase of cellular and mitochondrial de-repression', coincident with the sudden and discontinuous drop in growth rate, was the point at which cytochrome *P*-450 synthesis stopped immediately. In addition, when cells were shifted from fermentable to non-fermentable carbon source or vice versa the loss or synthesis of cytochrome *P*-450 was clearly seen.

The results presented here show that the amount of cytochrome *P*-450 was virtually independent of the sugar used for growth. A low amount of sugar did not, however, induce the synthesis of cytochrome *P*-450. Lactose and pentoses, which probably were not used by the yeast, did not cause cytochrome *P*-450 to appear. Thus the substrate availability *per se* was critical in the accumulation of cytochrome *P*-450. The disappearance of cytochrome *P*-450 began immediately when the cells were transferred from glucose to ethanol medium, which suggests that the repression is a response to a lowering of extracellular glucose concentration. The shift from ethanol to glucose, however, caused the synthesis of *P*-450 after a short lag period. The signal for the initiation of de-repression may require the accumulation of some catabolite during growth. All these observations suggest that the synthesis of *P*-450 in *S. cerevisiae* is caused by a common catabolite of the sugars, or an effector generated by this catabolite. This compound must be at a certain minimal concentration to induce the synthesis of cytochrome *P*-450.

The high mitochondrial cytochrome oxidase content of aerobically grown cells may interfere at 450nm in the spectrum of cytochrome *P*-450 when the measurements are made from whole yeast cells.

By mixing cell suspensions containing cytochrome *P*-450 (glucose-grown cells) with cell suspensions rich in cytochrome oxidase (ethanol-grown cells), it was shown that an amount of cytochrome *P*-450 of 0.018 nmol/mg of protein was clearly seen as a peak in the CO-difference spectrum, but an amount of 0.010 nmol/mg of protein was not detectable, due to the trough in the CO-difference spectrum at 450 nm caused by cytochrome oxidase. It seems also that cytochrome *P*-450 is more labile during aeration and its degradation rate is increased. Therefore the maximal amount during aerobic growth does not reach the same value as in non-aerated cultures, and the disappearance of the haemoprotein is faster after the sugar in the medium has been depleted.

The other peak observed in the reduced CO difference spectra between 400 and 500 nm, namely 'cytochrome *P*-420', occurred in all yeast cells whether grown on fermenting or non-fermenting energy sources. Its concentration was high also in those cases where the availability of substrate was very poor, in contrast with cytochrome *P*-450. It shows that at least some CO-binding pigments were formed also in such situations. It is possible that the 'cytochrome *P*-420' is in part a denatured form of cytochrome *P*-450 (Yoshida & Kumaoka, 1975; Yoshida *et al.*, 1977), at least in those cases where cytochrome *P*-450 has been accumulated. However, because the 420 nm-absorbing material accumulated in the absence of cytochrome *P*-450, when its maximal amount was in the exponential phase of growth, in contrast with its accumulation in the late stationary phase in cytochrome *P*-450-positive cells, it is not likely to be a denatured product of cytochrome *P*-450. It is, of course, possible that, under these different growth conditions, different pigments cause the peak at 420 nm (see Ishidate *et al.*, 1969a; Mok *et al.*, 1969). Because its concentration was higher in galactose- than in glucose- or fructose-grown cells and it accumulated earlier, it seems that its synthesis is related to the respiration condition.

The formation of a functional cytochrome *P*-450 requires the synthesis of the prosthetic group, haem, and the apoprotein, their assembly and subsequent integration of the product into the microsomal membrane. Haem is synthesized in both fermentative and respirative conditions (Labbe-Bois & Volland, 1977). It seems probable, therefore, that it is the protein component of cytochrome *P*-450 that is regulated in those different conditions.

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