Vol. 123, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1984 Pages 186-193

CYTOCHROME P-450 INDUCIBILITY BY ETHANOL AND 7-ETHOXYCOUMARIN O-DEETHYLATION IN S.cerevisiae

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Received July 11, 1984

SUMMARY: The level of cytochrome P-450 and some enzymatic activity cytochrome P-450 dependent in a diploid strain (D7) of S.cerevisiae are affected by the substrate supporting growth and its concentration and, in particular, by the growth phase of the culture. For these reasons we tested the hypothesis that the induction of the monooxygenase system in the D7 strain when grown in high concentration of glucose depended on one product of glycolysis, ethanol. There was a strict correlation between the level of cytochrome P-450 and the ethanol concentration. Moreover we developed a sensitive test measuring the ethoxycoumarin O-deethylation in order to detect the enzymatic activity cytochrome P-450 dependent in whole yeast cells, in different growth conditions.

Some strains of Saccharomyces cerevisiae contain a microsomal cytochrome P-450 dependent monooxygenase system very similar to that in mammals. The role of cytochrome P-450 in S.cerevisiae has not been completely clarified: yet it is known to be involved in the demethylation of lanosterol to ergosterol (1,2) and in the hydroxylation of benzo(a)pyrene only in fermentative conditions (3,4).

This enzymatic system of S.cerevisiae is also capable to metabolize promutagens such as dimethylnitrosamine, aflatoxin B, ß-naphthylamine, cyclophosphamide, perchloroethylene (5) and styrene (6) to their active mutagenic products. Recently we have also demonstrated in S.cerevisiae strain D7 the detoxification of 4-Nitroquinoline-1-oxide by cytochrome P-450 (unpublished results). The level of cytochrome P-450 in the D7 strain can be affected by inducers such as phenobarbital (7), by the type of carbon and energy source,

0006-291X/84 $1.50

its concentration, and by the growth phase of the culture. Thus we tested the hypothesis that the induction of the monooxygenase system in this strain in fermentative conditions and during the logarithmic growth phase was affected by one product of the glycolysis, i.e. ethanol. Ethanol is known to induce the monooxygenase system of mammals (10) and it has also been demonstrated that when yeasts are grown on a medium containing only ethanol as energy source, cytochrome P-450 is not found in the cells (8).

In the present investigation two series of experiments were carried out. First, we studied the effect of ethanol added at different concentrations (from 1% to 5%) in a liquid medium containing 2% glucose on the production of cytochrome P-450. Second, we examined the effect of growth phase on the production of cytochrome P-450 and of ethanol by yeast cells grown in liquid medium containing 20% glucose. In the same experiments, we measured the production of cytochrome P-450 and of ethanol by cells grown in liquid medium containing either 0.5%, 1% or 2% glucose.

To measure the monooxygenase activity at different growth phase and in the presence of different concentrations of glucose in the medium, we have developed a simple and sensitive method to determine in whole yeast cells the O-deethylation of 7-ethoxycoumarin to the higly fluorescent umbelliferon.

MATERIALS AND METHODS

Saccharomyces cerevisiae strain D7, obtained from Prof. F.K.Zimmermann. Mitotic gene conversion at the trp5 locus, point mutation of the mutant allele ilv-92 and mitotic recombination between the centromere at the ade2 locus (11) can be detected with this yeast strain.

Media: The liquid growth medium was made up of one liter of deionized water to which were added 10 g of yeast extract, 20 g of Bacto Peptone and either 20,2,1 or 0.5% of glucose. When ethanol was used for the induction of cytochrome P-450, the composition was the same except that only 2% glucose was included with either 1,1.5,2,3,4 or 5% ethanol.

Culture conditions: The initial inoculum was at a concentration of 150-300 cells in 100 ml of liquid medium. The cultures were incubated at 30°C.

Protein determination: Total protein concentration was determined according to Lowry et al (12) with bovine serum albumine as the standard.

Assay of cytochrome P-450 from whole cells: Samples corresponding to a cell concentration of about 10^9 were harvested by centrifugation for 10 min at 2000xg. They were resuspended after two washes in phosphate buffer (0.05M), pH 7,4 containing 0.1M EDTA. The measurements of cytochrome P-450 were carried out as described by Omura and Sato (16). Recordings of the different CO spectra were made in a scanning Perkin-Elmer spectrophotometer. A molar extinction coefficient of $91000 M^{-1} cm^{-1}$ was used for the cytochrome P-450, reduced and bound with CO minus reduced cytochrome P-450, between 450 and 490 nm.

Determination of ethanol concentration: Alcohol dehydrogenase was employed to measure the concentration of ethanol by the method of Bucher and Radetzki.(14)

Assay of deethylation of 7-ethoxycoumarin: On every harvested sample of whole cells, the deethylation of 7-ethoxycoumarin was determined as described by Ullrich and Weber (13). Samples corresponding to about $4x10^9$ cells were harvested, and after two washes, the cells were resuspended in 15 ml of 0.05M,Tris-HCl buffer, 1.15% KCl, pH 7.4. The incubation of 2.5 ml of cell suspension with 250 λ of ethoxycoumarin was performed at 37°C. After incubation for 5 min and 10 min, the reaction was terminated by addition of 0.6 ml 15% Trichloroacetic acid and 2.2 ml of 1.6 M, Glycine-NaOH buffer,pH 10.3. The fluorescent umbelliferon was measured at 440 nm with excitation at 390 nm in Perkin-Elmer 650-10S fluorescence spectrophotometer.

RESULTS

Table 1 shows the determinations of cytochrome P-450 in yeast cultures in the presence of different concentrations of ethanol added to the normal liquid medium containing 2% glucose. It was found that the concentration of cytochrome P-450 increased with increasing ethanol concentrations up to about 2%. At ethanol concentration of 4% and more the cytochrome P-450 induction appeared to be reduced or not detectable. In Table 2 are shown the determinations of ethanol and of cytochrome P-450 produced when the yeast cells were grown in liquid medium containing different concentrations of glucose and at different stages of growth.

Table 1. Level of cytochrome P-450 in cells of S. cerevisiae D7 strain, grown in liquid media containing 2%, 1%, 0.5% glucose and known ethanol concentration, at different growth phases

Conc. % P/V glucose	Conc.% V/V Ethanol	Cells/ml $\times 10^6$	pMol cyt.P-450 $\times 10^8$ cell
2	0.0	45	9±1.1
	1.0	48	10±0.4
	1.5	39	12±1.5
	2.0	45	19±2.3
	3.0	36	11±0.6
	4.0	67	2±0.2
	5.0	45	0.0
1	0.0	49	5±1.0
	2.0	49	10±1.2
0.5	0.0	36	0.0
	2.0	36	7±0.9

Results are given as means of 5 independent experiments ±SD

Table 2 Determination of ethanol concentration produced by S.cerevisiae D7 strain in the growth media containning 20%, 2%, 1%, 0.5% glucose at different growth phases

Conc.% P/V. glucose	Cells/ml $\times 10^6$	pMol cyt.P-450 $\times 10^8$ cell	Conc.% V/V ethanol
20	50	20±2.4	2.1±0.3
20	137	5±0.7	3.9±0.8
20	250	0.0	4.2±0.7
2	49	9±1.1	0.6±0.1
1	48	5±1.0	0.5±0.04
0.5	36	0.0	0.0

Results are given as means of 5 independent experiments ± SD.

The maximal concentration of cytochrome P-450, i.e. 0.02 nmol/10^8 cells, was reached in the 20% glucose liquid media cultures when there were about $50x10^6$ cells/ml. It is evident that there is a decrease of cytochrome P-450 when the ethanol concentration exceeds 2.1% in the liquid growth medium. Levels of ethanol below 1% v/v were determined in 1%, 2% and 0.5% glucose cultures corresponding to low levels of cytochrome P-450 in the case of 1% and 2% glucose, and undetectable levels in the case of 0.5% glucose. To assay and determine the drug-metabolizing activity of the yeast cytochrome P-450 in the experimental conditions described above, we developed a simple biochemical method. In Table 3 are reported the levels of 7-ethoxycoumarin deethylation by whole cells harvested at different phases of the growth. The corresponding values of cytochrome P-450 are reported. The maximal activity was found after 10 min of incubation by cells harvested during logarithmic phase when the cytochrome P-450 level was at its peak. A strict correlation between the level of cytochrome P-450 and the deethylation activity can be observed.

Table 3. Ethoxycoumarin O-deethylation by whole cells of S.cerevisiae D7 strain harvested at different growth phases

Cells/ml x 10^6	pMol cyt.P-450 /mg Tot.Prot.	Time of incubation (min)		
		0	5	10
23	0.0	0	0.0	0.0
50	13±1.8	0	8.9±1.39	12.3±1.51
60	4±0.8	0	1.2±0.11	2.4±0.46
150	0.0	0	0.0	0.0

Corresponding levels of cytochrome P-450 are reported.
The activity is expressed as $\frac{\text{nmol umbelliferon*}}{\text{mg tot.prot.x min.}}$

Results are given as means of 5 independent experiments ± SD.
* The total proteins were detected from the cells used for the assay and corresponded to 1.4 mg/10^8 cells.

DISCUSSION

The highest level of cytochrome P-450 in cultures of D7 strain was determined to be during the logarithmic growth phase. The lower levels of cytochrome P-450 were observed in liquid media containing 1% or 2% glucose, and no cytochrome P-450 was detected in medium containing 0.5% of glucose (Table 1).

We have demonstrated that low concentrations of ethanol (from 1% to about 2.5% v/v), added initially to the normal liquid medium containing 2% glucose, increased the level of cytochrome P-450 (Table 2), as compared to the low levels produced in 1% and 2% glucose medium without initial addition of ethanol.

At initial ethanol concentrations higher than 3%, an inhibition of cytochrome P-450 was observed. The clearest evidence of the induction of cytochrome P-450 by ethanol was that in liquid medium containing 0.5% glucose no cytochrome P-450 was found whereas it was possible to detect it when 2% ethanol was added initially to the medium.

Ethanol concentrations in the liquid medium were determined also during cell growth. It was found that the optimal concentration of ethanol (2.1%) for the inductive effect was reached when cultures grown in media containing 20% glucose had reached the density of about 50×10^6 cells/ml (Table 2). At higher cell densities, the ethanol concentration increased, apparently causing the inhibition of the synthesis of cytochrome P-450, as has been reported for mammalian systems (15). Since livers of ethanol pretreated rats had a higher specific activity for the O-deethylation of 7-etoxycoumarin (15), we investigated whether cells, grown in high glucose concentrations, could also perform during the logarithmic phase the deethylation of 7-ethoxycoumarin. A simple and sensitive method to test the activity using whole

cells was therefore developed. We found (Table 3) that yeast cells were capable of 7-ethoxycoumarin deethylation cytochrome P-450 dependent. The maximal activity was reached during logarithmic growth phase at a cell density of about $50x10^6$ cell/ml (Table 3). No activity was detected before this growth phase and a decrease was observed at higher cell densities. This pattern is closely correlated with the variations of the level of cytochrome P-450. On the contrary no aminopyrine demethylase activity was found during logarithmic growth either in whole cells or in microsome preparations. This could be due to a specific inductive effect of ethanol. Thus we propose the determination of deethylation of 7-ethoxycoumarin as a useful test to estimate the induction state of monooxygenase system in yeast cells.

ACKNOWLEDGEMENTS

We thank Prof. C. Bauer (our Institute) for interesting suggestions and Dr. D. Charles (Institut fur Genetik Neuherberg) for critical revising the manuscript.

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