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CUMENE HYDROPEROXIDE AND YEAST CYTOCHROME P-450: SPECTRAL INTERACTIONS AND EFFECT ON THE GENETIC ACTIVITY OF PROMUTAGENS

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SUMMARY:

Cells of Saccharomyces cerevisiae, harvested from log phase cultures, contain cytochrome P-450 and are capable of activating promutagens to products that are genetically active in the same cell. The effect of cumene hydroperoxide, a compound known to support cytochrome P-450-mediated reactions, on the activation of a variety of the promutagens was investigated. In all cases the genetic activity of the promutagens was increased. With dimethylnitrosamine as the promutagen, the increased rate of gene conversion was linear for at least 1 hr. Yeast cytochrome P-450 was stable in intact cells in the presence of cumene hydroperoxide. However, in microsomal preparations the cytochrome was rapidly destroyed. When cumene hydroperoxide was added to a suspension of intact yeast cells, a spectrum with a Soret maximum at 455 nm - indicative of an interaction with cytochrome P-450 - was observed.

INTRODUCTION:

The hepatic cytochrome P-450-dependent monooxygenase system is capable of metabolizing a variety of endogenous and exogenous substrates. This enzyme system requires NADPH and molecular oxygen for activity but these cofactors can be replaced *in vitro* by organic hydroperoxides (1-5). Cumene hydroperoxide has been found to be the most efficient of these compounds at mediating cytochrome P-450-catalyzed ractions (1). The rates of cumene hydroperoxide-mediated metabolism of many substrates are faster than the NADPH-dependent reactions (1, 3), but they lose linearity after a few minutes due to the rapid destruction of cytochrome P-450 (5, 6). Cells of the yeast Saccharomyces cerevisiae, harvested from log phase cultures with glucose as carbon source,

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contain cytochrome P-450 and are capable of metabolizing promutagens to products genetically active in the same cell (7). In this paper the effect of cumene hydroperoxide on this activation system is described.

MATERIALS AND METHODS:

Strains: To assess the genetic activity, the frequencies of trp5 and ade2 convertants were estimated in the diploid strain D4 of S. cerevisiae (8). The strain D5 (9) was used for the spectral analysis since long phase cells of this strain possess ten times more cytochrome P-450 (38.5 nmol per g dry wt) than the strain D4 and are also capable of activating promutagens (7).

Treatment of cells with promutagens and estimation of conversion frequencies: The method used has been described previously (7). Yeast cells were harvested from a log phase culture (this corresponds to the period of maximal cytochrome P-450 concentration) grown on complete medium with glucose as carbon source (2% peptone, 1% yeast extract, and 2% glucose). The cells at a concentration of 2 to 3 x 10^7 per ml were incubated with the promutagen in phosphate buffer, pH 7.0, at 37°C for varying times. Treatments were terminated by washing with ice-cold buffer. Appropriate aliquots of cells were plated on either minimal medium (0.6% Difco yeast nitrogen base without amino acids, 2% glucose) supplemented with tryptophan or minimal medium supplemented with adenine for the estimation of conversion frequencies and on minimal medium with both supplements for the estimation of survival. Complete details of the methods used for the estimations and the inherent variability encountered have been described (7).

Spectral analysis: Cells of the strain D5 were harvested from log phase cultures grown on complete medium with glucose as carbon source. Suspensions of 10^8 to 10^9 cells/ml in 0.1 M phosphate buffer, pH 7.0, were used. Yeast microsomes were prepared as described previously (7). Spectra were recorded with an Aminco DW2A spectrophotometer using stoppered, glass cuvettes with 1 cm pathlengths.

RESULTS AND DISCUSSION:

The effect of cumene hydroperoxide on the activity of various chemicals in the yeast strain D4 is given in Tables 1, 2, and 3. At a concentration of 1 mM, cumene hydroperoxide did not result in any appreciable decrease in cell survival when tested for up to 4 hr at 37°C (Tables 1, 2, and 3). An increase in the trp5 and ade2 conversion frequencies was sometimes observed but this increase was less than a doubling in the spontaneous frequencies and did not shown any relationship to duration of treatment. Concentrations of cumene hydroperoxide higher than 1 mM resulted in decreased cell survival but did not increase the conversion frequencies.

The effect of cumene hydroperoxide on the activity of dimethylnitrosamine in yeast cells harvested from log phase cultures (grown on medium containing glucose as carbon source) is given in Table 1. Treatment of cells for 60

Table 1. Effect of cumene hydroperoxide on the activation of dimethylnitrosamine by cells of the strain D4 harvested from medium with glucose as carbon source. Relationship to time of incubation.

Time (mins)	Concentration Dimethyl-nitrosamine (M)	Cumene hydroperoxide absent			Cumene hydroperoxide present (1mM)		
		survival (%)	Frequency of convertants per 10^5 survivors		survival (%)	Frequency of convertants per 10^5 survivors	
			Trp5	Ade2		Trp5	Ade2
15	0	N.D.			100	1.7	1.9
	0.11	91	1.1	1.3	89	1.8	2.4
	0.45	88	1.2	1.9	74	5.7	7.7
30	0	N.D.			100	1.5	2.1
	0.11	95	1.1	1.5	84	2.4	2.8
	0.45	82	1.6	2.1	63	10.8	13.8
60	0	N.D.			100	1.6	2.0
	0.11	100	1.3	1.4	91	2.1	2.5
	0.45	85	2.4	2.1	19	21.7	30.8
240	0	100	1.1	1.3	100	1.7	1.9
	0.11	91	1.2	1.7	74	2.7	2.5
	0.45	23	5.2	2.6	<0.1		

N.D. not determined. Treatments consisted of log phase cells (2 to 3 x 10^7 cells/ml) incubated at 37°C with various concentrations of dimethylnitrosamine.

min with dimethylnitrosamine (450 mM) resulted in an approximate doubling of the spontaneous conversion frequencies. Cumene hydroperoxide (1 mM) increased the conversion frequencies in cells treated with dimethylnitrosamine (450 mM) from 2-fold to 15-fold as compared to the spontaneous rates. These increases were linear with time. Cell survival was similar when the treatment consisted of dimethylnitrosamine (450 mM) alone for 4 hr or in combination with cumene hydroperoxide (1 mM) for 1 hr. However, in the former case the conversion frequencies were only 2- to 5-fold higher than the spontaneous rates.

The conversion frequencies in cells treated with aflatoxin B_1 for 30 min and β-naphthylamine, dimethylsulfoxide, or ethyl carbamate for 45 min were all significantly increased by the addition of cumene hydroperoxide to the incuba-

Table 2. Effect of cumene hydroperpxode on the activation of various promutagens by strain D4.

Promutagen	Conc. (mM)	Cumene hydroperoxide absent			Cumene hydroperoxide present (1mM)		
		survival (%)	Frequency of convertants per 10^5 survivors		survival (%)	Frequency of convertants per 10^5 survivors	
			Trp5	Ade2		Trp5	Ade2
Aflatoxin B_1	0	100	1.3	1.5	100	2.5	2.9
	0.27	91	2.4	1.9	83	10.1	12.3
	0.54	88	1.7	2.1	32	20.8	30.8
β-naphthyl-amine	0	100	2.2	1.5	100	3.3	2.5
	1.2	86	2.6	2.0	77	10.1	9.6
	2.3	35	5.9	4.2	8	19.5	14.1
Ethyl carbamate	0	100	2.2	1.5	100	3.3	2.5
	75.0	97	2.7	1.6	100	3.9	3.5
	150.0	80	2.9	2.1	10	8.3	9.2
Dimethyl-sulphoxide	0	100	1.1	1.1	100	1.2	1.8
	940.0	78	1.4	1.6	92	3.0	3.9
	1408.0	85	1.6	1.6	69	5.6	7.2

Incubations with aflatoxin B_1 were for 30 min. The other three compounds were incubated for 45 min. Incubations consisted of the compound and cells of the strain D4 harvested from log phase cultures grown on medium containing glucose as carbon source. Ethyl carbamate was added in aqueous solution, β-naphthylamine as a solution in ethanol and aflatoxin B_1 as a solution in a 1:1 mixture of ethanol and dimethylsulphoxide (the concentration of dimethylsulphoxide was less than 0.5 M.

tion mixtures (Table 2). The increased frequencies were similar to rates previously obtained by treatment of yeast cells for 4 hr with these compounds alone (7).

Cells harvested from cultures grown on galactose did not activate dimethylnitrosamine but were able to activate aflatoxin B_1 (7). These cells have a low concentration of cytochrome P-450, estimated from solubilized microsomes to be 0.05 nmol/10^{10}cells. However, the effect of these compounds on the galactose-cultured yeast cells was greatly altered by cumene hydroperoxide; significant numbers of dimethylnitrosamine-induced convertants were detected and the aflatoxin B_1-induced conversion frequencies were increased 4- to 5-fold (Table 3). Metabolism of aflatoxin B_1 is thought to be mediated by cytochrome P-450 and the observed effect of cumene hydroperoxide is consistent

Table 3. Effect of cumene hydroperoxide on the activation of aflatoxin B_1 and dimethylnitrosamine in cells of the strain D4 harvested from medium containing galactose as carbon source.

Promutagen	Conc. (mM)	Cumene hydroperoxide absent			Cumene hydroperoxide present (1mM)		
		survival (%)	Frequency of convertants per 10^5 survivors		survival (%)	Frequency of convertants per 10^5 survivors	
			Trp5	Ade2		Trp5	Ade2
Aflatoxin B_1	0	100	1.5	1.5	93	1.6	1.3
	0.27	100	6.4	6.1	80	15.3	13.8
	0.54	100	4.0	3.7	32	19.6	10.9
Dimethyl-nitrosamine	0	100	1.5	1.5	93	1.6	1.3
	315	93	1.8	1.7	51	5.6	2.2
	450	82	1.9	1.5	2	5.4	3.1

Treatments were incubated at 37°C for four hours.

with this hypothesis. Thus, the substrate specificities of the cytochromes found in cells cultured on glucose or galactose appear to be different.

In mammalian hepatic microsomal monooxygenase systems, the addition of cumene hydroperoxide results in a rapid rate of substrate metabolism which quickly loses linearity due to the destruction of cytochrome P-450 (5, 6). The addition of cumene hydroperoxide to yeast microsomes also resulted in the destruction of the cytochrome (Fig. 1). However, in intact yeast cells the cytochrome P-450 was stable for at least 1 hr in the presence of this compound. In addition, a stable cumene hydroperoxide-induced complex with cytochrome P-450 was observed (Fig. 2). The formation of the spectrum was time-dependent and reached a maximum after approximately 5 minutes. Isosbestic points were observed at 446 and 494 nm. The carbon monoxide-ferrous cytochrome P-450 difference spectrum obtained after the formation of the cumene hydroperoxide-induced complex was of the same magnitude as the spectrum obtained in the absence of the complex. Thus, cumene hydroperoxide appears to form a reversible complex with ferrous cytochrome P-450 in intact yeast cells without destroying the cytochrome. The ferrous state of the cytochrome in

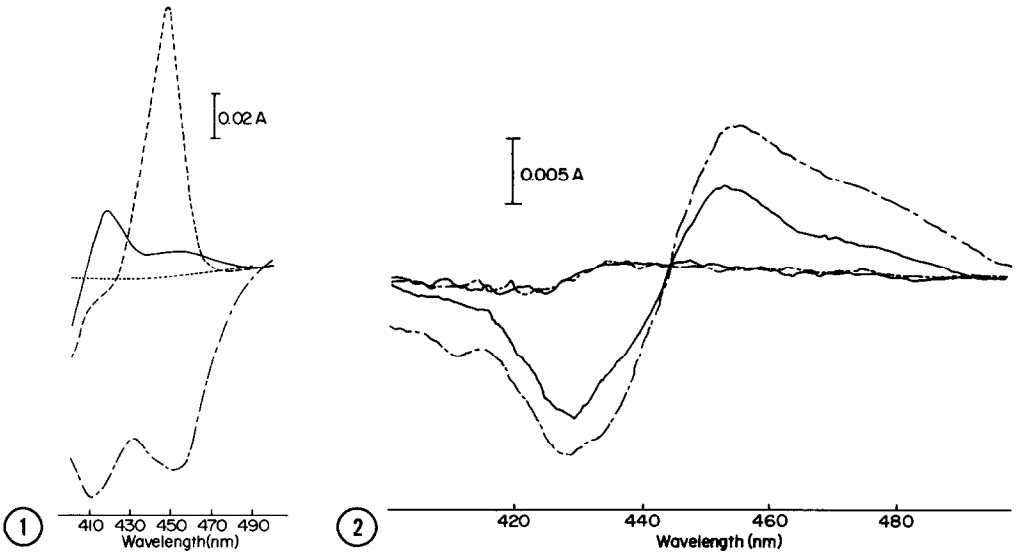

Figure 1. Destructive effect of cumene hydroperoxide on cytochrome P-450 in microsomes prepared from the yeast D5 strain.
————, CO-difference spectrum of dithionite-reduced microsomes after treatment of microsomes with cumene hydroperoxide (2 mM) for 15 minutes at 25°C. Both cuvettes contained cumene-treated microsomes and dithionite and the sample cuvette contained CO.
--------, CO-difference spectrum of dithionite-reduced microsomes. Both cuvettes contained untreated microsomes and dithionite and the sample cuvette contained CO.
– – – – , Cumene hydroperoxide difference spectrum of dithionite-reduced, CO-treated microsomes. Both cuvettes contained dithionite and CO and the sample cuvette contained cumene hydroperoxide (2 mM).
....., Baseline obtained with microsomes in both cuvettes.

Figure 2. Difference spectra obtained on the addition of cumene hydroperoxide (2 mM) to a suspension of yeast cells.
Yeast cells of the D5 strain were harvested from log phase cultures on complete medium with glucose as carbon source.
————, Experiment 1, the concentration of cytochrome P-450 was 0.82 nmol/ml.
– – – – , Experiment 2, the concentration of cytochrome P-450 was 1.3 nmol/ml.

intact yeast cells was confirmed by the formation of a maximum carbon monoxide-cytochrome P-450 complex on the addition of as little as 5 µl of a CO-saturated solution to a 3 ml suspension of cells in the absence of exogenous reducing agent.

The position of the Soret maximum at 455 nm in the cumene hydroperoxide-induced spectrum in intact yeast cells also suggested a complex with ferrous cytochrome P-450. Similar spectra have been described for the complexes of a

number of compounds with ferrous cytochrome P-450, for example, methylene-dioxyphenyls (10) and halogenoalkanes (11).

In conclusion, yeast provides a system for the study of cytochrome P-450 both *in vivo* and *in vitro*. The experiments reported here show that the use of cumene hydroperoxide greatly enhances the sensitivity of yeast systems used for the identification of promutagens. This is probably the case because cumene hydroperoxide increases the rate of activation of promutagens in yeast without destroying the cytochrome P-450.

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