Eur. J. Biochem. 36, 583–592 (1973)

Properties of a Yeast Cytochrome P-450-Containing Enzyme System which Catalyzes the Hydroxylation of Fatty Acids, Alkanes, and Drugs

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(Received January 1/April 10, 1973)

The growth of *Candida tropicalis* on tetradecane causes the induction of a cytochrome P-450-containing enzyme system which catalyzes the hydroxylation of fatty acids, hydrocarbons and drugs. When the cells are broken by treatment with a French pressure cell, the cytochrome P-450 is obtained in an apparently soluble form. The enzyme system was resolved into three components: cytochrome P-450, NADPH-cytochrome P-450 reductase, and a heat-stable lipid fraction, all of which are necessary, along with NADPH and molecular oxygen, for the conversion of laurate to ω-hydroxylaurate. NADH alone is almost completely inactive but causes a doubling of the activity when present along with a saturating level of NADPH. The yeast reductase and lipid fractions may be replaced by corresponding fractions obtained from rat liver microsomes. The effect of various phospholipids on the hydroxylation activity was investigated, and a yeast lysophosphatidylethanolamine fraction was shown to be most effective as judged by laurate hydroxylation. The yeast cytochrome P-450 is not readily autoxidizable, as shown by experiments in which it was reduced extensively by NADPH in the presence of phospholipid and the reductase under aerobic conditions.

Cytochrome P-450, which serves as a biological catalyst for the hydroxylation of a wide variety of substrates, occurs not only in microsomes or mitochondria from animal [1] and human tissues [2–4], but also in bacteria [5–7], bacteroids [8], and fungi [9]. Recent work in this laboratory [10,11] has shown that cytochrome P-450 is induced in strain LM7 of *Candida tropicalis* by growth on hydrocarbons under aerobic conditions and is functional in the hydroxylation of hydrocarbons, fatty acids and drugs. The only other yeast in which cytochrome P-450 has been found is *Saccharomyces cerevisiae*, which contains more of this hemoprotein when grown anaerobically or semi-anaerobically on carbohydrates than when grown aerobically [12 to 14]. The hydroxylating enzyme system of *C. tropicalis* has the properties of a mixed function oxidase in that it requires both NADPH and molecular oxygen for activity.

The present paper describes the resolution and partial purification of the cytochrome P-450 of *C. tropicalis* and the role of phospholipids in the hydroxylation activity of this enzyme. Some of the properties of the yeast enzyme system have been reported briefly [10,11], including its ability to hydroxylate a variety of substrates and its inhibition by carbon monoxide. The yeast cytochrome P-450 is similar in many respects to that in liver microsomes which also catalyzes fatty acid ω-hydroxylation [15–19].

MATERIALS AND METHODS

Culture of Yeast Strain

Strain LM7 of *Candida tropicalis*, which was kindly furnished by the British Petroleum Company (Lavera, France) utilizes long-chain *n*-alkanes as the major carbon source. *n*-Tetradecane was the best growth substrate of several odd- and even-numbered hydrocarbons tested. The strain was kept on slants containing Sabouraud maltose agar (made by dissolving 10 g of Neopeptone, 40 g of maltose, and 15 g of Bacto-Agar in 1 l of water) coated with a thin film of tetradecane, and the organism was transferred every four weeks. The cells from one slant were used to inoculate 1 l of liquid culture in a 2.5-l flask, which was shaken at 200 rev./min at 30 °C for 36 h. The composition of the medium, which is similar to that used by Lebeault *et al.* [20] for the growth of Strain 101 of *C. tropicalis*, is shown in Table 1. The cells from one such flask were used to inoculate 9 l of the same medium in a 14-l New Brunswick Microferm fermenter, and the culture

Table 1. *Growth medium for* Candida tropicalis *(strain LM 7)* The pH, after autoclaving, was 5.8, the temperature was 32 °C, and aeration was at the rate of 0.4 l per min per liter of medium

Component	Amount
	g/l
Tetradecane	5.0
Yeast extract	0.25
NH_4Cl	2.5
KH_2PO_4	7.0
Na_2HPO_4	1.2
$MgSO_4 \cdot 7\ H_2O$	0.2
NaCl	0.1
$CaCl_2$	0.05
$Fe(NH_4)_2(SO_4)_2$	(trace)
$CuSO_4$	(trace)
$ZnSO_4$	(trace)

was stirred at 1000 rev./min at 32 °C. The pH of the medium was maintained at 5.5 by the occasional addition of 7 N NH_4OH. At the end of the logarithmic growth phase (20—22 h) the suspension was centrifuged to give about 15 g of cells, wet weight, per liter of medium. The resulting paste was flushed with nitrogen and stored in the frozen state.

Solubilization and Resolution of Yeast-Enzyme System

Unless stated otherwise, all operations were carried out at 4 °C. The protein concentration of all preparations was determined by the method of Lowry *et al.* [21]. Thawed cells (15 g, wet weight) were suspended in 30 ml of 0.13 M sucrose containing 0.12 M potassium citrate buffer pH 7.4, 34% glycerol, and 0.1 mM dithiothreitol and passed through a French pressure cell with an outlet pressure of greater than 1500 kg/cm². An additional 30 ml of the same solution were added and the mixture was stirred for 15 min and centrifuged at 37000 × *g*. The sedimented material, which contained less than 5% of the total hydroxylation activity, was discarded. The supernatant fraction (65 ml), which contained about 12 mg protein and 1 to 2 nmol cytochrome P-450 per ml, was stored under nitrogen in the frozen state for several months without significant loss of activity. For resolution of the enzyme system, a 20-ml portion of this crude extract was put onto a DEAE-cellulose column (20 × 0.5 cm) previously equilibrated with 0.1 M Tris buffer pH 7.4, containing 0.1 mM dithiothreitol. The column was washed with 60 ml of the same buffer solution, and a protein fraction (Fraction A) was eluted which contained cytochrome P-450 (in 20% yield) and a smaller amount of cytochrome P-420. Attempts to decrease the conversion of cytochrome P-450 to P-420 by the addition of glycerol to the buffer solution were not successful. Fraction B, which had high NADPH-cytochrome *c* reductase activity, was eluted from the column with 100 ml of the same buffer solution containing 0.2 M KCl, and Fraction C, which contained a heat-stable lipid, was eluted with 0.5 M KCl. This procedure is useful in solubilizing and separating the components of the enzyme system but does not result in purification of the cytochrome P-450.

Partial Purification of Cytochrome P-450

Although cytochrome P-450 was solubilized without detergent treatment, as described above, a bile salt was included in the procedure for partial purification because it appeared to aid in the removal of phospholipids. To 10 ml of the extract, 11.5 ml of 0.13 M sucrose containing 0.12 M citrate buffer pH 7.4 and 0.1 mM dithiothreitol were added. A 10% sodium cholate solution was then added to give a final concentration of 1 mg/mg protein, and the mixture was stirred for 15 min and then centrifuged for 15 min at 37000 × *g*. The supernatant fraction was treated with ammonium sulfate, and the fraction that precipitated between 27 and 45% saturation was dissolved in 2 ml 0.1 M Tris buffer pH 7.4, containing 0.1 mM dithiothreitol and 20% glycerol. This solution contained about 0.4 nmol cytochrome P-450 per mg protein and was free of cytochrome P-420 and cytochrome b_5. The procedure results in a 5-fold purification of cytochrome P-450 in 80% yield from the crude extract and largely removes the reductase and the functional lipid. Attempts to purify the cytochrome P-450 by a variety of other procedures have so far been unsuccessful.

Assay of Fatty-Acid Hydroxylation

The enzymatic activity of the cytochrome P-450 preparations was tested in an assay mixture containing the following components in a total volume of 1.0 ml: 100 μmol Tris buffer pH 6.5, 0.2 μmol [1-^{14}C]laurate (90000 counts/min), 0.3 μmol NADPH, yeast cytochrome P-450 fraction (0.1 to 0.2 nmol), partially purified liver microsomal NADPH cytochrome P-450 reductase (0.08 mg protein), and liver microsomal lipid fraction (0.15 mg lipid). Higher concentrations of laurate were not used because they are somewhat inhibitory. The final pH of the reaction mixture was 7.0. The reaction was initiated by the addition of the radioactive substrate, and the mixture was incubated at 30 °C for 10 min. The reaction was stopped by the addition of 0.4 ml of 10% H_2SO_4, and the mixture was extracted with 5 ml of 9:1 benzene—ether. The radioactive ω-hydroxylaurate was isolated by silicic acid chromatography [22], and the radioactivity was determined in a scintillation counter. All assays were done in duplicate, and the data were corrected for the slight activity observed in control experiments with the cytochrome P-450 fraction omitted.

Assay of NADPH-Cytochrome c Reductase

The activity of the various yeast fractions in catalyzing NADPH-dependent cytochrome *c* reduction was estimated by a slight modification of the procedure of Masters *et al.* [23]. The reaction mixtures contained, in a total volume of 1.0 ml, 500 μmol potassium phosphate buffer pH 7.7, 0.1 μmol cytochrome *c*, the reductase sample, and 0.1 μmol NADPH as the last addition. The reaction was followed at 550 nm at 30 °C with a spectrophotometer equipped with a Gilford multiple-sample absorbance recorder. The rate of reduction was found to be constant for about 2 min.

Cytochrome P-450 Determination

The concentration of cytochrome P-450 was determined from the carbon monoxide difference spectrum. Carbon monoxide was bubbled through the solution for 30 s before the addition of a few grains of sodium dithionite. After 5 min the cytochrome P-450 concentration was calculated from the absorbance difference, $A_{450}-A_{490}$, on the assumption that yeast cytochrome P-450 has the same absorption coefficient as reported by Omura and Sato [24] for liver microsmal cytochrome P-450.

Thin-Layer Chromatography

Precoated Eastman silicic acid Chromagram sheets without fluorescent indicator were used for lipid separation with chloroform—methanol—glacial acetic acid—water (75:25:4:2, by vol.) as the solvent. In some experiments, in order to avoid tailing of phosphatidylserine and to achieve its separation from lysophospholipids, chloroform—methanol—concentrated ammonium hydroxide (75:25:4, by vol.) was used as the solvent.

Gas-Liquid Chromatography

For the identification of fatty acid esters, a 2.4-m column of 5% DEGS on Gas-chrom Q, 100 to 120 mesh, was used at 180 °C in an F & M Gas Chromatograph (Model 420) equipped with a flame ionization detector.

Isolation of Yeast Lipids

Lyophilized yeast cells (40 g) having a lipid content equal to about 10% of the total dry weight were extracted once with 10 volumes of absolute acetone and twice with 10 volumes of chloroform—methanol (2:1, v/v). The acetone solution was evaporated, and the residue was combined with the chloroform-methanol extracts and washed as described by Folch *et al.* [25]. After evaporation of the solvents, 4.0 g residue were obtained, 1.5 g of which were dissolved in 6 ml chloroform and applied to a Bio-Sil A column (25 × 2.2 cm) previously washed with chloroform—methanol (1:1, v/v) and then equilibrated with chloroform. Neutral lipids, fatty acids, and traces of hydrocarbon were eluted by 150 ml chloroform, and phospholipids were eluted by 120 ml each of 3:1, 2:1, and 1:1 (v/v) chloroform—methanol. Fractions of 20 ml each were collected, and the lipids present were identified by comparison with authentic compounds on silicic acid thin-layer chromatography plates in two different solvents. Phospholipids were detected on the plates by a molybdenum spray described by Dittmer and Lester [26] and lipids containing free amino groups by spraying with ninhydrin and heating. The major phospholipid was phosphatidylethanolamine, and smaller amounts of phosphatidylcholine, phosphatidylserine, and lysophosphatidylethanolamine were also isolated. The fractions containing the same phospholipids were pooled, and the solvents were removed in a rotary evaporator. The lipids recovered were suspended by sonication in 0.02 M phosphate buffer pH 7.4 and assayed for activity in the laurate hydroxylation system.

Materials

Technical grade *n*-tetradecane, which was used as the growth substrate for the yeast, was obtained from Phillips Petroleum; Bacto-yeast extract, Neopeptone, maltose, and Bacto-Agar from Difco, silicic acid (Bio-Sil A, 100—200 mesh) from Bio-Rad, NADH, NADPH, DEAE-cellulose, and Antifoam B from Sigma, and lipid standards of high purity from Supelco (Bellefonte, Pennsylvania). Liver microsomal NADPH-cytochrome P-450 reductase, partially purified as described previously [27] and the microsomal lipid fraction from DEAE-cellulose chromatography [15] were generously provided by Ms Joanne K. Heidema. [1-^{14}C]Lauric acid (specific activity, 31.2 mCi/mmol), bought from Amersham/Searle, was diluted with nonradioactive lauric acid and neutralized with KOH to give a 20 mM solution of potassium laurate. Since this concentration exceeds the solubility at room temperature, the mixture was warmed before use.

RESULTS

Factors Affecting Cytochrome P-450 Content of Yeast

Cytochrome P-450 is induced in cells of *C. tropicalis* by growth on tetradecane, but not by growth on glucose, as reported earlier [10]. Rapid growth of cells and maximal yield of cytochrome P-450 are dependent on a high speed of stirring, apparently because it ensures a good dispersion of the hydrocarbon, as shown by others [28]. Air was bubbled through the mixture at the rate of 0.4 l per min per liter of medium at the beginning of the fermentation, but the rate of aeration had to be decreased at

Table 2. *Yeast fractions required for laurate hydroxylation*
Fractions A, B, and C were present in the amounts of 0.57, 0.24, and 0.1 mg protein, respectively. In later experiments it was shown that the activity in Fraction C was due to the lipid present, rather than the protein. Laurate hydroxylation was assayed as described in the text

Fractions present	NADPH-cytochrome *c* reductase activity	Cytochrome P-450 content	Hydroxylation activity
	nmol × min^{-1} × mg protein^{-1}	nmol/mg protein	nmol/min
A	7	0.07	0.35
B	276	0	0.17
C	3	0	0.07
A + B			0.44
A + C			0.60
A + B + C			0.84

later stages because of foaming. This was not prevented by the addition of Antifoam B, possibly because of degradation of the emulsifier by the yeast. The use of this agent had the disadvantage that it caused an increase in the amount of cytochrome P-420 at the expense of cytochrome P-450. When oxygen was used instead of air, both the growth rate and the cytochrome P-450 content of the cells decreased. The cytochrome P-450 content was highest when the culture was harvested at the end of the logarithmic growth phase; the cell yield was 12—15 g, wet weight, per liter of medium. Longer growth times increased the yield of cells but resulted in increased formation of cytochrome P-420.

Resolution of Enzyme System

Cytochrome P-450 was readily solubilized from *C. tropicalis* as judged by the criterion that no precipitate formed when the crude extract was centrifuged for 1 h at 100000 × *g*. The enzyme system was resolved into its components by the method recently applied by Lu and Coon [15] to liver microsomes, but with deoxycholate and KCl omitted from the solution. As described above, this method yielded three fractions, which were tested for activity as shown in Table 2. The results show that cytochrome P-450 was present primarily in Fraction A and the reductase primarily in Fraction B. Cytochrome *c* was used as an artificial electron acceptor for the reductase because of the convenience of the assay, and it was assumed that, as with the corresponding reductase from liver microsomes, cytochrome P-450 is the natural acceptor. Maximal hydroxylation activity was obtained when all three fractions were present. The stimulation in activity due to the addition of Fraction C was still seen when it had been boiled for 2 min, and the activity was shown to be extractable into 2:1 chloroform—methanol. Thin-layer chromatography of this extract showed the presence of phospholipids.

Table 3. *Activity of yeast cytochrome P-450 in presence of rat liver microsomal phospholipid and reductase*
The assay was carried out as described in the text with partially purified yeast cytochrome P-450 (0.23 nmol, 1.24 mg protein) and the usual amounts of the microsomal reductase and lipid fractions

System	Hydroxylation activity
	nmol/min
Complete	4.00
No reductase	0.20
No lipid	0.48
No cytochrome P-450	0.24

Activity of Yeast Cytochrome P-450 in Presence of Liver-Microsomal Reductase and Various Phospholipids

Because the yeast system showed obvious similarities to the liver microsomal hydroxylation system, attempts were made to couple the partially purified cytochrome P-450 fraction with the reductase and lipid fractions from rat liver microsomes, with the results shown in Table 3. This combination of yeast and microsomal components was highly effective. The apparent activity, determined at saturating levels of the reductase and the phospholipid, was about 17 mol product formed per mol cytochrome P-450 per min, which is greater than found for laurate hydroxylation by solubilized rabbit liver microsomal cytochrome P-450. With cytochrome P-450 from some batches of yeast cells, however, the apparent activity was as low as 3; the cause of this variation is not known. In other experiments the yeast lipid fraction (Fraction C) was substituted for the liver microsomal lipid, with a resulting activity 80% as great.

Laurate hydroxylation in the coupled enzyme system was linear with time for at least 10 min, as indicated in Fig. 1. The rate of hydroxylation was proportional to the concentration of each of the components of the enzyme system when the other two were present at saturating concentrations (Fig. 2). Thus, each component was readily assayed when tested under suitable conditions in this procedure. Each of the three components became somewhat inhibitory when added at higher levels than shown. Fig. 3 shows the activity of the coupled enzyme system in 0.1 M Tris and phosphate buffers at various pH values. The highest laurate hydroxylation activity was at about pH 7.0 in Tris buffer and pH 6.8 in phosphate buffer; the maximal rate was somewhat greater in Tris buffer. In other experiments it was found that when the concentration of the Tris buffer at pH 7.0 was increased to 0.5 M, the activity dropped to 60%, and when increased to 0.7 M only 25% of the original activity remained.

Identification of Reaction Product

Various reports indicate that fatty acids undergo biological hydroxylation at the ω-1 carbon atom [29—31] as well as at the ω-position [15, 16, 22, 30—32]. To determine which compounds are formed in the yeast system, 10 typical assay mixtures containing radioactive laurate were incubated for 10 min at 30 °C, and the reaction products were combined after chromatographic separation from the unreacted substrate. Authentic ω-hydroxy acid (2 μmol) and dodecandioic acid (2 μmol) were added, and the mixture was separated on a thin-layer chromatography plate using petroleum ether—ether—acetic acid (40:60:6, by vol.) as solvent. The material in the areas corresponding to lauric acid (R_F 0.75), the dioic acid (R_F 0.55), and the hydroxy acid (R_F 0.42) was scraped off the plate and counted in a scintillation counter after the addition of thixotropic gel. More than 90% of the radioactivity recovered was associated with the hydroxy-fatty acid and about

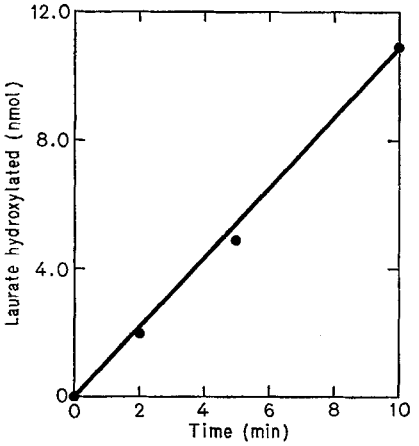

Fig. 1. *Laurate hydroxylation as a function of time.* The yeast cytochrome P-450 fraction (0.29 nmol, 0.93 mg protein) was used with the microsomal reductase and lipid fractions

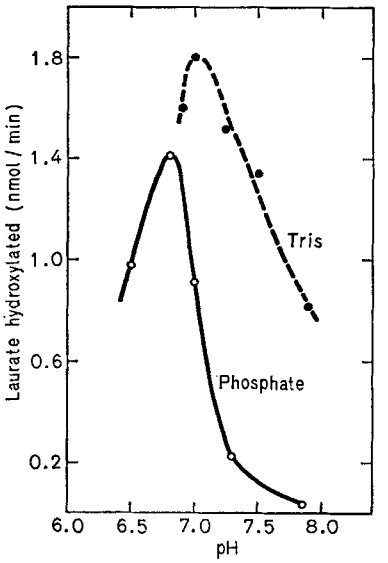

Fig. 3. *Laurate hydroxylation in the coupled enzyme system as a function of pH.* Each assay mixture contained 100 μmol Tris chloride or potassium phosphate buffer, with yeast cytochrome P-450 (0.23 nmol, 1.2 mg protein) and other components as described in the text. The pH values were measured in the final reaction mixtures at 30 °C

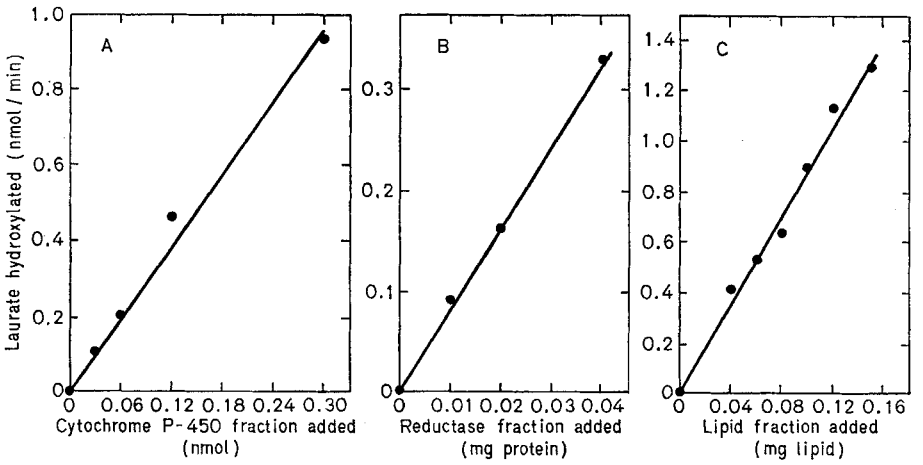

Fig. 2. *Laurate hydroxylation as a function of concentration of components of enzyme system.* In Experiment A, the microsomal reductase fraction (0.08 mg protein) and microsomal lipid (0.15 mg) were in excess and the yeast cytochrome P-450 was varied. In Experiment B, the cytochrome P-450 fraction (0.13 nmol, 0.80 mg protein) and lipid (0.15 mg) were in excess and the reductase was varied. In Experiment C, the cytochrome P-450 fraction (0.09 nmol, 0.85 mg protein) and reductase fraction (0.08 mg protein) were in excess and the microsomal lipid was varied. The data are corrected for product formation in the absence of the component being varied: 0.05, 0.02, and 0.1 nmol in A, B, and C, respectively

5% with the dicarboxylic acid. With a 30-min incubation time in place of the usual 10 min, the amount of dicarboxylic acid increased to 16%. Presumably the dioic acid had been formed by dehydrogenation of the ω-hydroxy acid. For further identification, the hydroxy-fatty acid derived from laurate was eluted from a thin-layer chromatography plate with 2:1 chloroform—methanol and esterified with BF_3-methanol according to Metcalfe and Schmitz [33]. After treatment with *N,O*-bis(trimethylsilyl)acetamide the mixture was submitted to gas-liquid chromatography, by which the ω- and (ω-1)-hydroxy fatty acid derivatives are easily distinguished [30]. The single sharp peak which appeared was symmetrical and had a retention time of 7.8 min, as did that from authentic ω-hydroxy acid. For further characterization the ω-hydroxylauric acid methyl ester was oxidized with CrO_3 as described by Kusunose *et al.* [22], converted to the dimethyl ester, and submitted to gas-liquid chromatography. A peak with a retention time the same as that of authentic dodecandioic ester (25.6 min) was the only compound detectable. The yield was about 90%. Therefore, ω-hydroxylation of lauric acid appears to be the major reaction in the enzyme system containing the yeast cytochrome P-450, and ω-1 hydroxylation, if it occurs at all, must be a minor reaction.

Effect of Various Phospholipids on Hydroxylation Activity

Various lipids were tested for activity in the laurate hydroxylation assay, with the results given in Fig.4. The yeast lysophosphatidylethanolamine was the most active, whereas the phosphatidylserine from the same source was only slightly over half as effective, and the yeast phosphatidylethanolamine was much inferior. The liver microsomal lipid fraction and a 5:1 mixture of synthetically prepared di- and monolauroylglyceryl-3-phosphorylcholine were fairly active at low concentrations but strongly inhibitory at higher levels. In other experiments not shown, di- and triglycerides were inactive, and synthetically prepared 1-linoleoyllysophosphatidylethanolamine (kindly furnished by Dr W. E. M. Lands) appeared to be highly active. When the isolated yeast lysophosphatidylethanolamine fraction was transesterified with BF_3-methanol according to the method of Morrison and Smith [34] and the fatty acid esters were analyzed by gas-liquid chromatography, it was found that 51% of the fatty acids were unsaturated, with linoleic acid being the most predominant. It may be noted that commercially available phospholipids from bacterial and animal sources were considerably less active in the hydroxylation system than the corresponding phospholipid fractions isolated from the yeast.

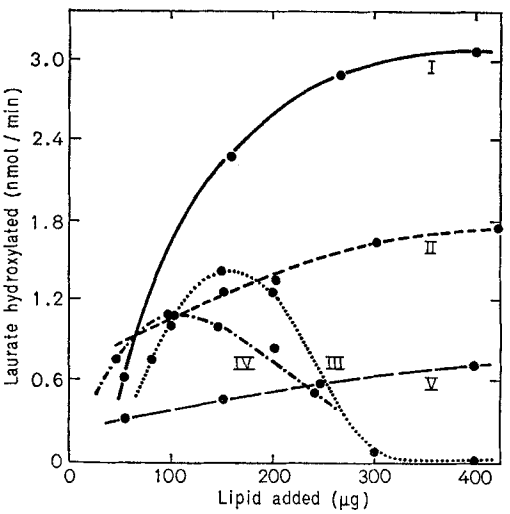

Fig.4. *Effect of various lipids on laurate hydroxylation.* Synthetic lauroylphosphatidylcholine and lipids isolated from yeast and from liver microsomes were assayed at different concentrations with yeast cytochrome P-450 (0.17 nmol) in the presence of the microsomal reductase fraction (0.08 mg protein) and the other usual components. The lipids (with the exception of the microsomal lipid fraction) were sonicated in a minimal volume of 0.02 M phosphate buffer pH 7.7 and added to the reaction mixture prior to the addition of the enzyme fractions. The order of addition did not appear to be critical. (I) Yeast lysophosphatidylethanolamine; (II) yeast phosphatidylserine; (III) microsomal lipid fraction; (IV) lauroylphosphatidylcholine; (V) yeast phosphatidylethanolamine

Properties of Yeast Cytochrome P-450

When the partially purified cytochrome P-450 preparation was submitted to disc gel electrophoresis on 4% polyacrylamide, two main protein bands were seen, along with several minor bands. Staining according to the method of Haut *et al.* [35] showed a single heme-containing band at the location of the main protein band of higher mobility. The total heme in the preparation was determined by the pyridine-hemochromogen method [36] and found to correspond closely to the cytochrome P-450 content. No cytochrome b_5 could be detected by the spectrophotometric method of Omura and Sato [37] in the presence of added NADH and partially purified rat liver NADH-cytochrome b_5 reductase. Of the total iron content, which was determined by the method of Ballentine and Burford [38], only 10% was accounted for by the cytochrome P-450, and the remainder represents unidentified nonheme iron. (We are grateful to Dr Robert M. Kaschnitz for these determinations.) It may be noted that nonheme iron is also present in larger amounts than heme iron in partially purified cytochrome P-450 preparations from rabbit liver microsomes [39].

In contrast to the liver microsomal cytochrome P-450, which is rapidly autoxidizable, the reduced

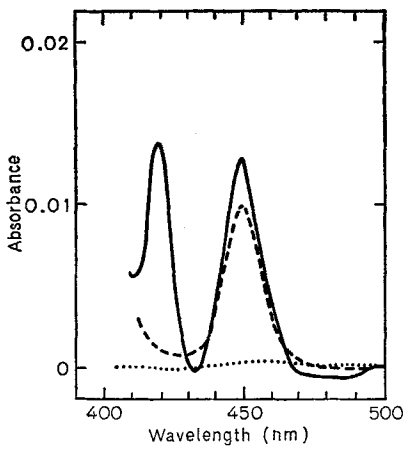

Fig. 5. *Reduction of yeast cytochrome P-450 under aerobic conditions as judged by formation of CO complex.* The yeast cytochrome P-450 fraction (0.14 nmol, 0.7 mg protein) was reduced in the presence of the microsomal reductase (0.08 mg protein) and microsomal lipid (0.1 mg) by 0.3 μmol NADPH, and the CO difference spectrum was recorded in a cuvette having a 1-cm light path. After several minutes, when the reaction was complete, a few grains of dithionine were added to both cuvettes. (·····) No reductant added; (– – – –) NADPH added; (——) dithionite added after NADPH

yeast pigment is stable in the presence of oxygen. Indeed, the cytochrome P-450 in the crude yeast extract was found to be completely reduced, possibly because of the presence of endogenous substrates capable of acting as reducing agents. The expected CO difference spectrum was obtained when the partially purified cytochrome P-450 preparation was reduced by dithionite in the presence of carbon monoxide. Complete reduction of the cytochrome P-450 by dithionite required about 5 min, but no cytochrome P-420 could be detected in some of the enzyme preparations, even at longer times. A similar experiment was carried out with a different sample of the cytochrome P-450 known to contain cytochrome P-420, with the results shown in Fig. 5. Enzymatic reduction by NADPH in the presence of the microsomal reductase and lipid fractions, although more rapid than reduction by dithionite, required several minutes, but incubation for as long as 30 min did not further increase the height of the peak at 450 nm. The apparent absence of cytochrome P-420 may be noted. However, when dithionite was then added, a small increase in reduced cytochrome P-450 was observed along with the appearance of reduced cytochrome P-420. In other experiments the omission of the phospholipid fraction from the reaction mixture resulted in the reduction of only 27% as much cytochrome P-450. These results show that the yeast cytochrome P-450 is reduced almost as extensively by the enzymatic system as by dithionite and that the enzymatically reduced cytochrome P-450 is not measurably autoxidizable. The yeast cytochrome P-420, on the other hand, is reduced by dithionite but not by NADPH under aerobic conditions, and therefore appears to be autoxidizable. This finding is supported by other experiments which showed that under anaerobic conditions the cytochrome P-420, as well as the cytochrome P-450, was readily reduced by NADPH in the presence of the reductase.

Pyridine-Nucleotide Requirement

In the hydroxylation of laurate by the yeast enzyme system, as reported earlier [10], NADPH is much more active than NADH. Apparently, therefore, the yeast reductase has the same pyridine nucleotide specificity as the microsomal reductase. When the partially purified yeast cytochrome P-450 was coupled with the liver microsomal reductase and lipid, the omission of NADPH or the substitution of NAD^+, NADH, or $NADP^+$ for NADPH at 0.3 mM, a known saturating concentration for NADPH, gave barely significant activity. The results obtained at higher NADH concentrations and with a combination of the reduced nucleotides are shown in Fig. 6. At the highest concentration tested, NADH was about 30% as effective as a saturating concentration of NADPH. On the other hand, with a combination of the two reduced pyridine nucleotides, a synergistic effect was noted. This was shown when NADPH was held at a fixed concentration (0.3 mM) and NADH was varied, or when NADH was held at a fixed concentration (2.0 mM) and NADPH was varied. The data presented clearly show that in the presence of both reduced cofactors the activity was about twice as great as would be predicted if their activities were only additive. It may be noted that NADPH was at a saturating concentration during the course of the reaction, as shown by the linearity of laurate hydroxylation with time (Fig. 1). Furthermore, since in various experiments NADH was added at concentrations up to 10 times that of NADPH, it seems highly unlikely that the individual reduced pyridine nucleotides were at rate-limiting concentrations. From the inverse plots in Fig. 7 the apparent K_m of NADPH was determined to be 0.32 mM and that of NADH 16 mM.

Substrate Specificity

Laurate is used routinely as the substrate for this enzyme since it is the most active of a series of compounds tested [10]. The apparent K_m of laurate, determined from rates measured with concentrations of 0.20 mM and lower, was found to be about 0.3 mM; however, concentrations above 0.20 mM proved to be inhibitory in the coupled enzyme system. For example, the activity at 0.30 mM

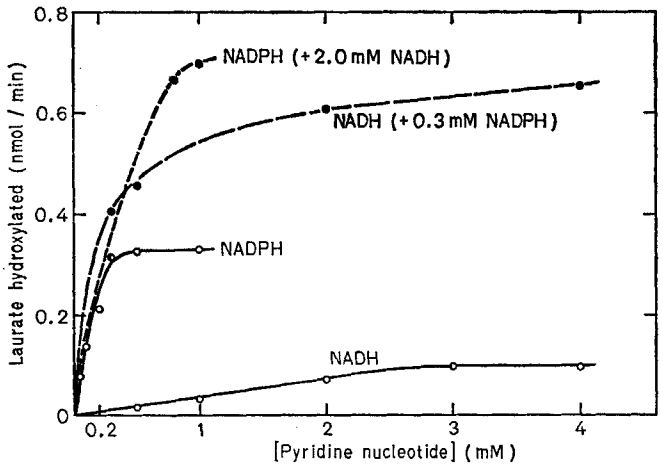

Fig. 6. *Synergistic effect of NADH and NADPH on laurate hydroxylation.* Cytochrome P-450 was present in the amount of 0.12 nmol along with the other components of the coupled system, including the reductase fraction in the amount of 0.08 mg protein and the concentration of NADPH or NADH was varied as shown in the curves with solid lines. The curves with dashed lines show the results obtained when the concentration of NADPH was varied in the presence of 2.0 mM NADH or the concentration of NADH was varied in the presence of 0.3 mM NADPH

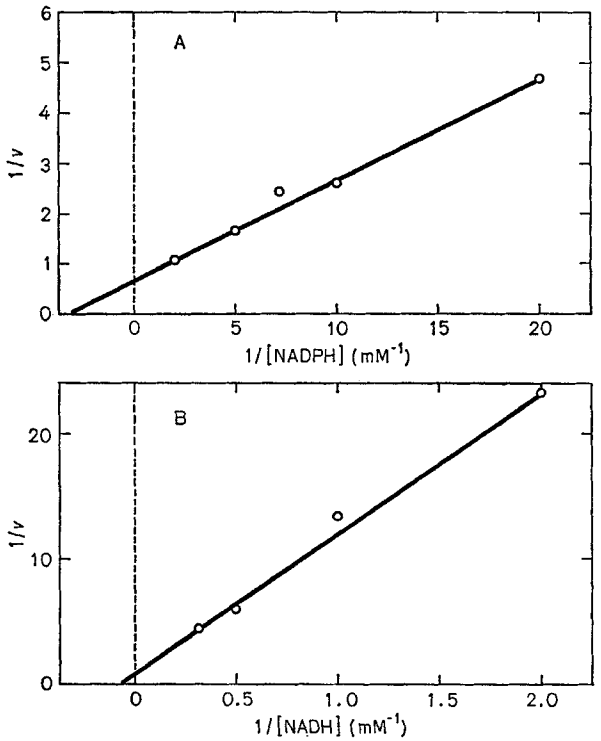

Fig. 7. *Determination of* K_m *of (A) NADPH and (B) NADH in the coupled enzyme system.* The yeast cytochrome P-450 fraction was present in the amount of 0.12 nmol (0.43 mg protein). Velocities are expressed as nmol product formed × min^{-1} mg $protein^{-1}$ in the cytochrome P-450 fraction and concentrations as molarities

Table 4. *Inhibition of laurate hydroxylation*
The various compounds were preincubated for 15 min at 0 °C with all of the components present except laurate. The reaction mixtures were then brought to 30 °C and radioactive laurate was added to initiate the reaction. Yeast cytochrome P-450 was present in the amount of 0.18 nmol (0.74 mg protein)

Compound added	Final concn	Inhibition
	mM	%
KCN	1.0	10
	5.0	56
	10	90
EDTA	5.0	16
	10	28
o-Phenanthroline	0.1	13
	0.5	60
	1.0	80
	3.0	93
8-Hydroxyquinoline	0.1	23
	0.6	52
	1.0	76
NaN_3	0.1	35
	1.0	52
	10	82

was only about 70% as great as at 0.20 mM. Other fatty acids were less active, and *n*-alkanes were found to be less active than fatty acids having the same number of carbon atoms [10]. The hydroxylation of drugs containing *N*-methyl groups was determined from the formaldehyde liberated spontaneously from the resulting *N*-hydroxymethyl compounds. The method of Nash [40] as modified by Cochin and Axelrod [41] was used for estimating the formaldehyde. With the partially purified cytochrome P-450 the relative rates, determined at the optimal concentration of each substrate, were as follows: laurate (0.20 mM), 100%; aminopyrine (1.0 mM), 45%; benzphetamine (1.0 mM), 24%; ethylmorphine (1.0 mM), 4%. The only *O*-methyl compound tested, *p*-nitroanisole, which is demethylated by the reconstituted rabbit liver microsomal enzyme system [42], was inactive with the yeast cytochrome P-450.

Effect of Inhibitors

As reported earlier, the laurate hydroxylation reaction catalyzed by the yeast cytochrome P-450 is inhibited by carbon monoxide [10]. When cyanide was added at concentrations up to 10 mM immediately before the reaction was initiated, no inhibition was observed, but when it was preincubated with the system for 15 min at 0 °C, the inhibition was extensive (Table 4). When preincubated in the same way, *o*-phenanthroline, 8-hydroxyquinoline,

and sodium azide gave strong inhibition, whereas EDTA was only partially inhibitory at the concentrations tested.

DISCUSSION

The growth of *C. tropicalis* on hydrocarbons under aerobic conditions induces a cytochrome P-450-containing enzyme system that hydroxylates a variety of fatty acids, hydrocarbons and drugs. Others have also observed the presence in yeast of pigments capable of combining with carbon monoxide. Lindenmayer and Smith [12], who first described the occurrence of a "450-CO" pigment in *S. cerevisiae*, found that its concentration was greater in cells grown anaerobically than under aerobic conditions. Ishidate *et al.* [13] reported a decrease in the cytochrome P-450 content of *S. cerevisiae* when cells grown anaerobically or semianaerobically on glucose were subsequently exposed to oxygen, and Yoshida *et al.* [43] have recently achieved the partial purification of cytochrome P-450 from microsomes of semianaerobically grown *S. cerevisiae*. From spectral evidence for the interconversion of the low-spin and high-spin states of the homoprotein they have concluded that it is similar to liver microsomal cytochrome P-450. There is no evidence available as yet, however, that cytochrome P-450 produced by the anaerobic or semianaerobic growth of yeast on carbohydrates is functional in hydroxylation reactions.

The cytochrome P-450 of *C. tropicalis* is located in the microsomal fraction of the cells according to the work of Gallo *et al.* [44], but it is solubilized more easily than the corresponding pigment of liver microsomal membranes. Whereas treatment with deoxycholate in the presence of stabilizing agents was found to be necessary for the solubilization and resolution of the liver enzyme system [15], that of *C. tropicalis* was solubilized and resolved, as described in the present paper, in the absence of detergents. The requirement for phospholipid for hydroxylation activity in the reconstituted yeast system is of special interest in view of the proposal of Cater *et al.* [45] that the function of the phospholipid in the liver microsomal system is to overcome inhibition caused by the detergent.

Recent investigations by Autor *et al.* [46,47] have shown that the phospholipid does not cause the resolved liver microsomal P-450 to form membrane-like aggregates, nor does it alter substrate binding [27, 48]. The phospholipid is required for the rapid phase of electron transfer to cytochrome P-450, as determined by stopped flow measurements [39,49—51], and it also governs the extent of reduction. Furthermore, the phospholipid must be present for substrate hydroxylation to occur when superoxide-generating systems are substituted for NADPH and the reductase in a reaction mixture containing the liver microsomal cytochrome P-450 [52—56]. In the yeast enzyme system, as shown by the results presented, the phospholipid is also required for hydroxylation and for extensive reduction of cytochrome P-450. Preliminary experiments which are not presented indicate that artificial or substitute reducing systems do not couple with the yeast cytochrome P-450 in the presence of phospholipid.

The requirement for NADPH and an NADPH-cytochrome P-450 reductase in liver microsomal hydroxylation and demethylation reactions is well established. Bacterial and mitochondrial hydroxylations involving cytochrome P-450 as a terminal oxidase require, in addition to a reduced pyridine nucleotide and a flavoprotein reductase, a nonheme iron protein. The *C. tropicalis* enzyme system exhibits a requirement for NADPH and the reductase, but a requirement has not been observed for an additional protein-containing fraction. Hildebrandt and Estabrook [57] showed that mixed function oxidation reactions in liver microsomes are stimulated by the addition of both NADPH and NADH, and they suggested microsomal drug metabolism may involve NADH, NADH-cytochrome b_5 reductase and cytochrome b_5 under certain conditions. On the other hand, Ichikawa and Loehr [58] have recently shown that the reduction of cytochrome P-450 in liver microsomal particles may occur in the absence of cytochrome b_5. The synergistic effect of NADH and NADPH in laurate hydroxylation would suggest a similar mechanism in this organism. Although the partially purified cytochrome P-450 fraction of yeast is apparently free of cytochrome b_5, small amounts are provided by the liver microsomal reductase fraction used, which contains about 0.2 nmol cytochrome b_5 per mg protein. Therefore, in the experiments in which the synergistic effect of NADH and NADPH was noted, cytochrome b_5 was present in the amount of 0.01 nmol/ml, or at about 1/10 the concentration of cytochrome P-450.

This research was supported by Grant GB-30419X from the National Science Foundation and Grant AM-10339 from the United States Public Health Service.

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