FATTY ACID AND HYDROCARBON HYDROXYLATION IN YEAST: ROLE OF CYTOCHROME P-450 IN *Candida tropicalis**

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Summary: A soluble enzyme system which catalyzes the conversion of fatty acids to their ω-hydroxy derivatives and n-alkanes to primary alcohols has been obtained from a strain of *Candida tropicalis* grown on tetradecane as the carbon source. The hydroxylation reaction requires TPNH and molecular oxygen and is inhibited by carbon monoxide. Lauric acid appeared to be the most active of a series of substrates tested. Fractionation of a cell-free extract of the yeast with ammonium sulfate indicated that at least two components are required for maximal activity. One fraction contained cytochrome P-450 as judged by the carbon monoxide difference spectrum of the dithionite-reduced preparation. The hydroxylation activity and the content of both cytochrome P-450 and TPNH-cytochrome c reductase (which may function as a cytochrome P-450 reductase) were greatly enhanced in cells grown on hydrocarbon.

Although many examples are known in which yeasts utilize hydrocarbons for growth or metabolism (1-7), in no instance is the nature of the alkane-oxidizing enzyme known. The present paper provides evidence that cytochrome P-450 is an essential component of the enzyme system in *Candida tropicalis* which catalyzes the hydroxylation of hydrocarbons and fatty acids.

The carbon monoxide-binding pigment of microsomes, called P-450 by Omura and Sato (8), is known to be a component of mixed function oxidases in animal tissues which catalyze the hydroxylation of steroids and the oxidative

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demethylation of drugs (9-11). Recent studies in this laboratory have led to the solubilization of the liver microsomal enzyme system and resolution into three components: cytochrome P-450, a reductase, and phosphatidylcholine, all of which are essential for the hydroxylation of alkanes and fatty acids as well as a series of drugs (12-17). Cytochrome P-450 has also been identified as a functional component in camphor hydroxylation in highly purified enzyme preparations from Pseudomonas putida (18,19) and in octane oxidation in extracts of a Corynebacterium (20,21).

C. tropicalis was grown in 10-liter batches in a New Brunswick Microferm Fermentor at 32° with tetradecane as the carbon source as described by Lebeault et al. (22). Air was passed through the culture at a rate of 12 liters per min with stirring at a rate of 200 rpm. The harvested cells (20 to 25 g, wet weight) were washed three times with 0.9% NaCl and then suspended in the medium used by Lu and Coon (12) to solubilize the hepatic microsomal enzyme system, except that deoxycholate was omitted. The suspension (40 ml final volume) was passed twice through a cold French pressure cell at an outlet pressure of greater than 10,000 psi. The preparation was diluted with an equal volume of the same medium and centrifuged for 20 min at 27,000 x g, and the sediment was discarded. The supernatant layer or "extract" was used directly in the experiments described or, in some instances, diluted with 4 volumes of 0.1 M Tris buffer, pH 7.7, and fractionated with solid ammonium sulfate. Fraction A refers to the protein precipitating between 0 and 40% saturation and Fraction B to that precipitating between 45 and 80% saturation. The enzyme system is apparently non-particulate since upon centrifugation of the crude extract for 90 min at 100,000 x g over 95% of the hydroxylation activity remained in the supernatant fraction.

The extract obtained was found to hydroxylate alkanes and fatty acids at the methyl carbon atom. Thus, when radioactive hexadecane was incubated with the enzyme preparation in the presence of TPNH, the products were identified by thin layer chromatography in 40:10:1 petroleum ether-ether-

acetic acid as hexadecanol (R_f 0.19) and palmitic acid (free acid, R_f 0.32; methyl ester, R_f 0.61). Radioactive laurate, under similar conditions, yielded products which were recovered by chromatography on silicic acid and identified by thin layer chromatography of the methyl esters in 70:30 petroleum ether-ether as ω-hydroxylaurate (R_f 0.10) and dodecandioate (R_f 0.41).

As shown in Table I, TPNH is required for laurate hydroxylation and cannot be replaced by TPN, DPNH, or DPN. The reaction also requires molecular oxygen, and it is inhibited by carbon monoxide. In other experiments cyanide (1.0×10^{-3} M) was found not to be inhibitory. Evidence is also given showing that at least two enzyme fractions are necessary for the hydroxylation reaction to occur at a maximal rate. The bulk of the hydroxylation activity

Table I

Requirements for laurate hydroxylation

Expt. No.	System	Gas phase	% Maximal activity[a]
1	Complete	Aerobic	100
	No TPNH	"	9
	TPNH replaced by TPN	"	9
	TPNH replaced by DPNH	"	8
	TPNH replaced by DPN	"	6
2	Complete	Aerobic	100
	"	Anaerobic	5
	"	$CO-O_2$ (1:2)	56
	"	$CO-O_2$ (2:1)	27
3	Complete (Fractions A and B)	Aerobic	100
	" (Fraction A)	"	29
	" (Fraction B)	"	6

The complete reaction mixture contained 100 μmoles of Tris buffer, pH 7.5, 0.5 μmole of TPNH or other pyridine nucleotide, 0.4 μmole of laurate-1-^{14}C (6.0×10^5 cpm), enzyme extract in Experiments 1 and 2 (2.1 and 2.6 mg of protein, respectively), and enzyme fractions in Experiment 3 (Fraction A, 0.5 mg of protein; Fraction B, 0.2 mg of protein). The final volume was 1.0 ml and the incubation was for 15 min at 30°. The radioactive ω-oxidation products formed were isolated by silicic acid chromatography.

[a]In the complete system the yield of products in the three experiments was 60, 81, and 8.1 nmoles, respectively.

precipitated between 40 and 45% saturation with ammonium sulfate, and no stimulation could be seen when that fraction was combined with either the 0-40% or the 45-80% fraction. On the other hand, about three-fold stimulation occurred when Fractions A and B were combined. Although the activity was not maximal due to the necessity of excluding the most active fraction, the experiment clearly established the need for two distinct components.

The specificity of the enzyme system is shown in Table II. Of a series of fatty acids tested, all served as substrates, and laurate proved to be the most active. Of the hydrocarbons tested, hexadecane appeared to be about 25% as active as laurate, and octane and decane were significantly active but less effective substrates. In other experiments drugs known to undergo N-

Table II

Substrate specificity

Compound tested	Concentration	Specific activity[a]
	M	
Laurate	5.0×10^{-4}	3.1
Myristate	5.0×10^{-4}	2.8
Undecanoate	5.0×10^{-4}	2.3
Decanoate	5.0×10^{-3}	2.2
Palmitate	5.0×10^{-4}	1.2
Stearate	5.0×10^{-4}	0.9
Octanoate	5.0×10^{-2}	0.8
Hexanoate	2.5×10^{-2}	0.5
Hexadecane	5.0×10^{-4}	0.8
Decane	5.0×10^{-4}	0.2
Octane	5.0×10^{-4}	0.2

The complete reaction mixture was like that in Table I, but with various substrates at the concentrations indicated. Fatty acids were added as the potassium salts and the products were recovered by column chromatography on silicic acid (23). Octane was added in acetone and decane in ethanol so that the final concentration of the solvent in the reaction mixture was 1%; the products were recovered by column chromatography on alumina (24). Hexadecane was added in ethanol and the products were recovered by thin layer chromatography on Silica Gel G plates in 40:10:1 petroleum ether (b.p. 30-60°)-ether-acetic acid.

[a] Product formed (nmoles) per min per mg of protein.

demethylation in liver microsomal preparations were tested with the yeast system. The demethylation reaction was determined by the formation of formaldehyde (25,26). The specific activities were as follows: aminopyrine, 0.9; benzphetamine, 0.5; hexobarbital, 0.5; and ethylmorphine, 0.2 nmoles of product formed per min per mg of protein.

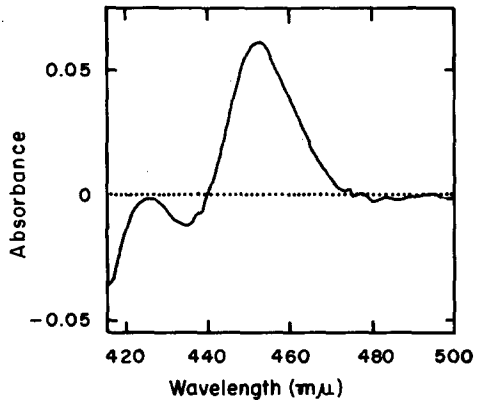

Fig. 1. Carbon monoxide difference spectrum of soluble cytochrome P-450 in extract of C. tropicalis grown on tetradecane. The preparation, containing 13.4 mg of protein per ml, was reduced by the addition of a few grains of solid dithionite. The sample cell was bubbled with CO for about 30 sec, and the CO difference spectrum was recorded in a Cary model 14 spectrophotometer with cuvettes of 1-cm light path.

In attempts to identify the enzyme component which is inhibited when it reacts with carbon monoxide, evidence was obtained for the presence of cytochrome P-450. As shown in Fig. 1, the carbon monoxide difference spectrum of the dithionite-reduced preparation, with a peak at about 450 mμ, is typical of this pigment. In other experiments the cytochrome P-450 was shown to be present in Fraction A but could not be detected in Fraction B (cf. Table I). Studies on the inducibility of the enzyme system are summarized in Table III. Cytochrome P-450 could be detected only in extracts of cells grown on hydrocarbon. Furthermore, both the TPNH-cytochrome c reductase activity, which is assumed to be a measure of TPNH-cytochrome P-450 reductase, and the hydroxylation activity were markedly higher in cells grown on hydrocarbon.

Table III

Inducibility of enzyme system by growth on tetradecane

Carbon source for growth	Cytochrome P-450 content	Reductase activity	Hydroxylation activity
	(nmoles)	(nmoles per min)	(nmoles per min)
Glucose[a]	0.00	32	0.1
Tetradecane	0.05	175	3.9

The cytochrome P-450 content was estimated from the CO difference spectrum on the assumption that the extinction cofficient of 91 cm^{-1} mM^{-1} for $A_{450}-A_{490}$ (27) also applies to the yeast pigment. TPNH-cytochrome c reductase was estimated spectrophotometrically (14) and laurate hydroxylation as in Table I. All values are expressed per mg of protein, determined by the method of Lowry et al. (28).

[a] Prior to the experiment with glucose, the cells were transferred ten times on agar slants containing glucose.

Others have observed the presence in yeast of pigments capable of combining with CO but having no known function. In 1964, Lindenmayer and Smith (29) described the occurrence of "450-CO" and "420-CO" pigments when S. cerevisiae was grown aerobically or anaerobically. More recently, Ishidate et al. (30) reported that the hemoprotein responsible for the 450 mμ maximum was more concentrated in semi-anaerobically grown cells and could be recovered in a particulate fraction.

The data in the present paper clearly indicate that cytochrome P-450 is a functional component of the inducible fatty acid- and hydrocarbon-hydroxylating enzyme system of C. tropicalis. Presumably this pigment has a similar function in other yeasts, but this remains to be established. It may be noted in this connection that Heinz et al. (31) recently reported the conversion of oleate to 17-hydroxyoleate in extracts of a species of Torulopsis and observed that the system was sensitive to carbon monoxide. In contrast to the results obtained with C. tropicalis, the enzyme system in Pseudomonas oleovorans which catalyzes the ω-hydroxylation of fatty acids and alkanes is inhibited by cyanide but not by carbon monoxide, and cytochrome P-450 is not present (32,33).

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