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Cytochromes P-450 of Yeasts

OTHMAR KÄPPELI

Institute of Biotechnology, Swiss Federal Institute of Technology Hönggerberg, 8093 Zurich, Switzerland

INTRODUCTION

The quantitative importance of oxidation reactions in the elimination of drugs in humans became apparent in the late 1940s and early 1950s. The enzymatic basis of this elimination process was established in 1963, when Creaven and Williams (24) demonstrated that subcellular fractions from human liver catalyzed the oxidation of biphenyl and coumarin. The initial short report was followed 3 years later by a paper by Kuntzman et al. (65) that characterized the drug-oxidizing system in human liver in detail. Activity was shown to reside in the microsomal fraction and to require reduced nicotinamide adenine dinucleotide phosphate (NADPH). In the following year Coccia and Westerfeld (23) reported that fractions of human liver were active in catalyzing many different routes of oxidation with chloropromazine as substrate.

In 1958, Klingenberg and Garfinkel (33, 63) independently announced the discovery of a carbon monoxide-binding pigment (hemoprotein) in the microsomal fraction of rodent liver. Later on, Omura and Sato (86) suggested that the heme nature of this porphyrin was of the *b* type. They also proposed the tentative name cytochrome P-450, which means "a pigment with an absorption at 450 nm in the reduced CO difference spectrum." Although this name is inconsistent with the system of nomenclature adopted for cytochromes, it was generally accepted.

Cytochromes have been divided into four groups according to the nature of the heme prosthetic group. Each of the four heme types, *a*, *b*, *c*, and *d*, has a different side chain in its porphyrins (Fig. 1). Well-established cytochromes are identified by subscript numerals with the letter indicating the groups, e.g., cytochrome b_5. The designation of cytochromes which are not well established is presently based on the wavelength of the α band (in nanometers) of the reduced form, e.g., cytochrome *c*-554 (28).

When cytochrome P-450-containing microsomes were treated with detergents with the aim of solubilizing the pigment, a new peak appeared at 420 nm in the reduced CO difference spectrum. Omura and Sato (86) concluded that this new peak was another form of cytochrome P-450 which retained the ability to combine with carbon monoxide in its

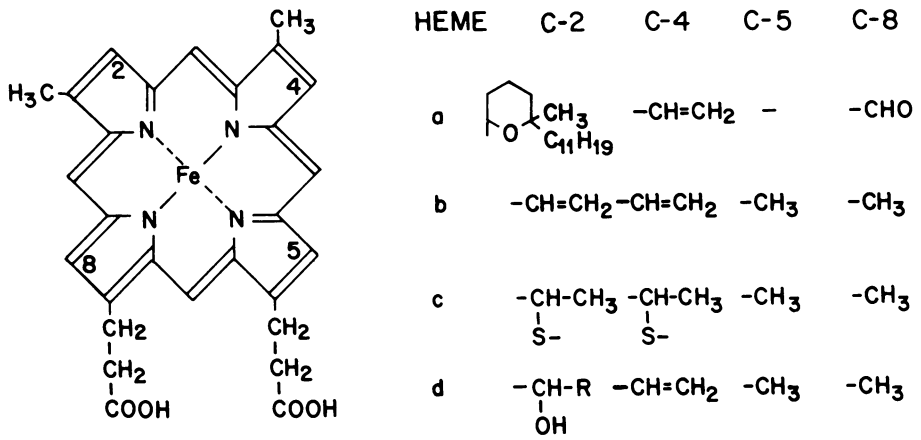

FIG. 1. Heme prosthetic groups of hemoproteins (51).

reduced state. The solubilized form was named cytochrome P-420 to distinguish it from the native, catalytically active, membrane-bound form.

Cytochrome P-450 Reactions

Cytochromes P-450 are the terminal oxidases of a variety of biotransformation systems used by most organisms. These terminal oxidases use molecular oxygen and electrons supplied by NADPH or NADH via a flavoprotein, cytochrome P-450 reductase. Other electron transport proteins are also present in some systems. In spite of the recent advances in the study of microsomal cytochrome P-450 regulation, purification, and chemical characterization, the mechanistic details of the hemoprotein catalytic cycle remain an open question. This is not a trivial problem since it involves the interaction of the hemoprotein with an electron donor, molecular oxygen, and a variety of apparently unrelated organic substrates (among others, fatty acids, alkanes, steroids, drugs, and xenobiotics), resulting in a wide diversity of oxidation products, some of which are still a challenge to synthetic organic chemistry. During catalysis, cytochrome P-450 not only overcomes the low kinetic reactivity of molecular oxygen, but also oxidizes carbon-hydrogen bonds with a variable degree of regio- and stereochemical selectivity according to the overall stoichiometry:

$$H^+ + RH + NADPH + O_2 \rightarrow ROH + NADP^+ + H_2O \quad (1)$$

It has been known for several years that cytochrome P-450, in addition to its oxygenase activity (reaction 1), also catalyzes the NADPH-dependent reduction of molecular oxygen to H_2O_2 (34, 41) as well as the lysis of oxygen-oxygen bond of some organic hydroperoxides (82). The stoichiometries of these so-called oxidase and peroxidase activities are given by reactions 2 and 3, respectively:

$$H^+ + NADPH + O_2 \rightarrow NADP^+ + H_2O_2 \quad (2)$$

$$R_1OOH + RH \rightarrow ROH + R_1\,OH \quad (3)$$

The above-mentioned reactions were summarized in a catalytic cycle by Capdevila et al. (22). The cytochrome P-450 reactions proposed illustrate the mechanistic versatility of the microsomal cytochrome P-450 enzyme system and speak against a unique mechanism of oxygenation.

Distribution of Cytochrome P-450 Enzymes in Nature

Cytochrome P-450 enzymes were first detected in liver microsomes, and it was initially thought that they were present in hepatic tissues only (90). However, cytochrome P-450 was susbsequently discovered in the mitochondria of adrenal gland (37) and was also found to be involved in mitochondrial steroid synthesis in many tissues (90). Cytochrome P-450 was also found in eucaryotic microbes, initially in the yeast *Saccharomyces cerevisiae* (68) and later in the bacterium *Rhizobium japonicum* (13). Since then cytochrome P-450 has been detected in many other microbial species. Müller and co-workers in 1984 (78) listed 41 microbial cytochrome P-450 systems. Subsequently, cytochrome P-450 was discovered in tissues of various vertebrates, invertebrates, and plants (85). It follows that a widespread distribution of cytochrome P-450 enzymes occurs in nature, demonstrating the important metabolic role of these systems in the physiology of life.

During recent years many genes coding for cytochrome P-450 enzymes have been cloned from different organisms, and the deoxyribonucleic acid sequences have been compared. Because of the sequence homology observed it has been postulated that all existing cytochrome P-450 enzymes could have had a common ancestor millions of years ago (30, 61, 80, 81). With cytochrome P-450 from *Pseudomonas putida* taken as the reference point, several conserved regions can be identified in enzymes originating from rodent or even human liver (30). Good homology, for example, is found in the alignment of sequences surrounding conserved cysteines thought to be involved in chelation of the heme iron of cytochrome P-450. This indicates that the structural integrity of the catalytic sites must be maintained.

Since the discovery of cytochrome P-450, the liver system has been studied most intensively. The different enzymes in the liver can be divided into two groups. The first group forms the broad specificity enzymes which are responsible for the detoxification of drugs and xenobiotics. Their functions consist of helping to convert such compounds to a form that can be more readily excreted from the body. The enzymes of this group also have endogenous functions, particularly in the biosynthesis of biologically active agents, including steroid hormones and the prostaglandins. These apparently constitutive cytochrome P-450 species occur in mouse and rat liver in at least four to seven forms. Each of them metabolizes a variety of compounds, with a greater specificity for certain compounds than for others, but with varying degrees of overlap of substrate specificity (79).

The second group consists of the inducible (79) or narrow-specificity (101) cytochrome P-450 forms. These enzymes are thought to be responsible for more effective metaboliza-

tion of foreign compounds entering the organism from the environment. The constitutive cytochrome P-450 forms metabolize the foreign chemicals to a certain degree before the induced enzymes are available. It is not clear how many forms of inducible liver cytochrome P-450 exist. It has been postulated that the cytochrome P-450 systems may be as important in defending the body against foreign chemicals as the immune system is in dealing with foreign pathogens (70). Paradoxically, however, some of the foreign chemicals, which initially are incapable of causing cancer, are converted to active carcinogens by cytochrome P-450-catalyzed reactions.

Practical Applications

Due to the wide range of compounds known to be cytochrome P-450 substrates, several areas of practical interest can be envisaged for cytochrome P-450. They include the following. (i) Selective hydroxylation or side chain cleavage reactions of organic compounds (75) on a preparative scale might be useful for the pharmaceutical industry. The stereospecificity of enzymatic reactions might be applied to steroid hydroxylation but also to the production of speciality chemicals (e.g., chiralic building blocks). The chemical hydroxylation of the aromatic ring is generally an inherently expensive step in the synthesis of an aromatic speciality chemical. This expense often results from the nonspecificity of the hydroxylation reaction, which forms unwanted by-products and is therefore an inefficient use of the starting material. Additional processing may be required to remove the by-products and to dispose of them properly. Chemical hydroxylations also require severe reaction conditions and therefore consume a large amount of energy. In addition, chemical reactions can result in the formation of undesirable contaminants. One highly publicized case is the dioxin contamination that occurs during the chemical synthesis of 2,4,5-trichlorophenoxyacetic acid, a herbicide and a component of Agent Orange. For these reasons biological activation of oxygen and its subsequent insertion into organic compounds currently attract a great deal of interest.

(ii) Cytochrome P-450 may be used in vitro to analyze the metabolic pattern of a prospective drug to synthesize and investigate the metabolites with respect to possible mutagenic or carcinogenic activities. This application of cytochrome P-450 is today already realized in the Ames test (5), but improvements of the currently applied technique which involves incubation of the chemicals with a liver fraction and subsequent mutagenicity testing with *Salmonella typhimurium* can be envisaged.

(iii) The ability of the cytochrome P-450 system to catalyze the conversion of drugs and other foreign substances into water-soluble compounds which can be excreted by the kidney opens the possibility of using these enzymes for an extracorporal metabolic detoxification of blood in the case of drug overdosage or other toxic damages (75).

(iv) Use of cytochrome P-450 as a test system for antifungal agents, since many of them are cytochrome P-450 inhibitors (99), is a possibility. Therefore, cytochrome P-450 may be used for the design of new antifungal compounds, and the exact mode of action may be investigated with isolated cytochrome P-450.

The many possible applications of cytochrome P-450 require the production of this enzyme in large amounts. This is certainly best achieved when microbial cytochrome P-450 can be used since mass culture of microorganisms is possible. To evaluate the possibility of substituting mammalian cytochrome P-450 with one of microbial origin the characteristics of the systems must be available. Here, an account is given of the current knowledge of yeast cytochrome P-450 enzymes and their potential in conducting typical reactions.

TABLE 1. Hydrocarbon-degrading and cytochrome P-450-containing yeasts[a]

Yeast species	Reference
C. tropicalis LM7	67
C. tropicalis 101	32
C. guilliermondii	97
	72
C. tropicalis ATCC 32167	35
C. pulcherima	95
C. maltosa	96
L. elongisporus	73
E. lipolytica	25
Torulopsis sp.	40
T. candida	46

[a] The hydroxylation of the *n*-alkanes is catalyzed by a cytochrome P-450 monooxygenase, which is induced by the substrate.

OCCURRENCE OF CYTOCHROME P-450 IN YEASTS

In 1964 Lindenmayer and Smith (68) demonstrated that cellular extracts of *S. cerevisiae* contain cytochrome P-450. Although they recognized the similarity of this yeast hemoprotein with liver microsomal cytochrome P-450, the detection was not followed immediately by further investigations. Ishidate et al. (49, 50) resumed the work on *S. cerevisiae* and showed that the occurrence of cytochrome P-450 in these yeasts is coupled to an intermediary state at the transition from aerobic to anaerobic metabolism, so-called semianaerobic conditions.

Later, cytochrome P-450 was detected in hydrocarbon-degrading yeasts of the genus *Candida* (67). In these yeasts cytochrome P-450 was induced by the hydrocarbon substrate. With glucose as the carbon source it was not detected in whole cells even under oxygen limitation. As a consequence, the question of the distribution of cytochrome P-450 in yeasts arose. It was not clear whether it was related to specific functions or whether it generally occurred in these eucaryotes.

Distribution and Induction of Cytochrome P-450

Hydrocarbon-assimilating yeasts. In hydrocarbon-utilizing yeasts, the induction of cytochrome P-450 by the substrate is generally accepted (Table 1). No hydroxylation system other than the cytochrome P-450-dependent monooxygenase has been reported. In a feed-controlled continuous cultivation, cytochrome P-450 content of the cells was linearly related to the substrate uptake rate (Fig. 2). Gmünder et al. (35) concluded from this behavior that the primary hydroxylation of the substrate may be the rate-limiting step in hydrocarbon degradation.

Glucose was found to act as a repressor of the system (71, 94–96). Further, derepression of the synthesis by oxygen limitation was also noticed. Gmünder et al. (35) observed derepression in *Candida tropicalis* below an oxygen partial pressure of 4 kPa (ca. 20% of air saturation). When the oxygen supply was further decreased, the cellular cytochrome P-450 content increased from 35 to 80 pmol (mg of dry cell weight)$^{-1}$. Similar behavior was reported for *C. guilliermondii*. Derepression of cytochrome P-450 biosyn-

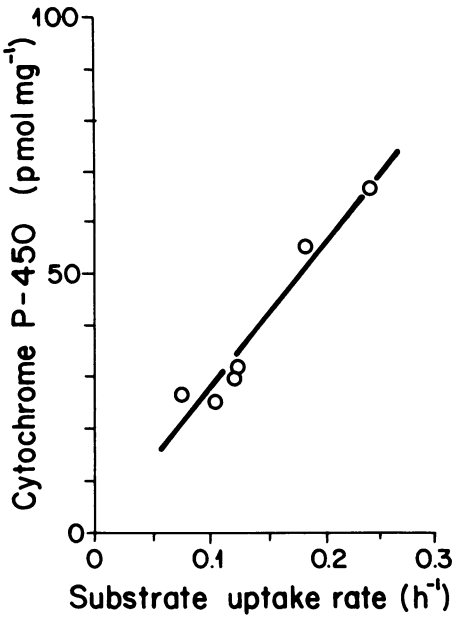

FIG. 2. Cytochrome P-450 content of *C. tropicalis* growing on hexadecane as a function of specific substrate uptake rate. The linear relationship indicates that the primary hydroxylation might be the rate-limiting step in the assimilation of hydrocarbons (35).

thesis occurred below a partial pressure of approximately 4 kPa. An increase of cytochrome P450 content from 30 to about 90 pmol (mg of dry cell weight)$^{-1}$ was observed with this yeast when the conditions were changed from carbon to oxygen limitation (72).

Cytochrome P-450 of *S. cerevisiae*. The formation of cytochrome P-450 in *S. cerevisiae* clearly depends on entirely different factors than in alkane-assimilating yeasts. It is characterized by a special metabolism when growing with glucose. The types of glucose metabolism encountered are listed in Table 2. Yeasts of the genus *Saccharomyces* are facultative anaerobes and their metabolism is modified depending on cultivation conditions (O. Käppeli, Adv. Microb. Physiol., in press). The occurrence of cytochrome P-450 in *Saccharomyces*-type yeasts is limited to the physiological state of fermentation. Figure 3 indicates that the respiratory glucose catabolism needs to be minimized (low specific oxygen uptake rates) and the fermentative one maximized (high specific ethanol production rates) to obtain high cellular cytochrome P-450 content. Generally, this is achieved by reducing respiration by oxygen limitation.

TABLE 2. Types of glucose utilization by *S. cerevisiae* in batch and continuous cultures

Conditions	Type of glucose catabolism
Batch culture	
Aerobic	
Growth phase 1	
C limited	Respirofermentative
O limited	Respirofermentative
Growth phase 2	Assimilation of ethanol
Anaerobic	Fermentative
Continuous culture	
Aerobic	
Low dilution rates	
C limited	Respirative
O limited	Respirofermentative
High dilution rates	
C limited	Respirofermentative
O limited	Respirofermentative
Anaerobic	Fermentative

For batch cultures Wiseman and King (101) reviewed the conditions which give rise to an enhanced expression of cytochrome P-450 in the genus *Saccharomyces*. They indicate that one of the following conditions is required: (i) high concentration of glucose (4 to 20%) in the growth medium; (ii) anaerobic growth conditions; (iii) addition of inhibitors of mitochondrial protein biosynthesis to the growth medium (including erythromycin and chloramphenicol); and (iv) use of respiratory-deficient mutants. These factors were also discussed in relation to the likely presence of low concentrations of cyclic adenosine 5′-monophosphate in the yeast cells (100, 103). The main effect of such conditions applied to yeasts is, however, a decreased synthesis of mitochondrial cytochromes. Several groups have noted an inverse relationship between mitochondrial cytochrome content (particularly cytochrome *a*) and cytochrome P-450 content of the cells. High cytochrome P-450 contents are obtained when the mitochondrial cytochrome concentration decreases (Fig. 4). Also, the influence of chloramphenicol, an inhibitor of mitochondrial protein synthesis, could be demonstrated most impressively in continuous-culture experiments (Fig. 5). Cytochrome *a* and *b* biosyntheses were inhibited by chloramphenicol, whereas cytochrome P-450 could be detected only in cells growing in the presence of chloramphenicol.

From the data recorded with whole cells (Fig. 5) the question arose of cytochrome P-450 content of cells with fully developed mitochondria. The problem originates from the fact that the spectra of cytochrome P-450 and mitochondrial cytochrome oxidase interfere in the reduced CO difference spectrum (56). Cytochrome oxidase has a trough at 441 to 445 nm, and therefore cytochrome P-450 may be hidden in cells with high mitochondrial cytochrome contents. We have addressed this problem in the search for cytochrome P-450 in yeasts other than those that are hydrocarbon degrading and of the *Saccharomyces* type.

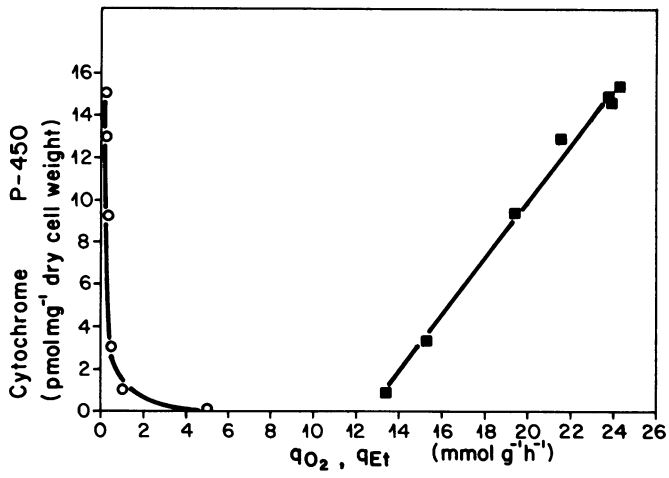

FIG. 3. Cytochrome P-450 content of *C. tropicalis* growing on glucose as a function of specific oxygen uptake rate (q_{O_2}) and specific ethanol production rate (q_{Et}). Minimum oxidative and maximum fermentative metabolism is required for high cytochrome P-450 contents of the cells, which are achieved under oxygen-limited growth conditions. Symbols: ○, q_{O_2}; ●, q_{Et}.

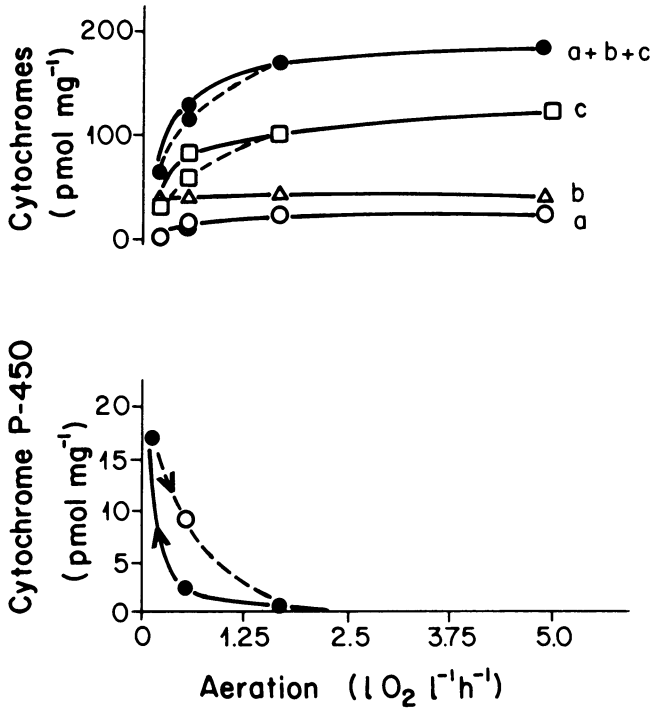

FIG. 4. Inverse relationship between mitochondrial and cytochrome P-450 contents of cells of *S. cerevisiae*. Cytochrome P-450 content increases when mitochondrial cytochrome contents decrease (98).

Cytochrome P-450 of other yeasts. Not all yeasts exhibit the same variety in glucose metabolism as *S. cerevisiae*. There are yeasts that degrade glucose by respiration only (e.g., *Trichosporon cutaneum* [52]) or form ethanol from glucose under oxygen-limited conditions only (e.g., *C. tropicalis*). For the investigation of the degree of distribution of cytochrome P-450, yeasts of different metabolic types were chosen (Table 3). Only in whole cells of alkane-grown

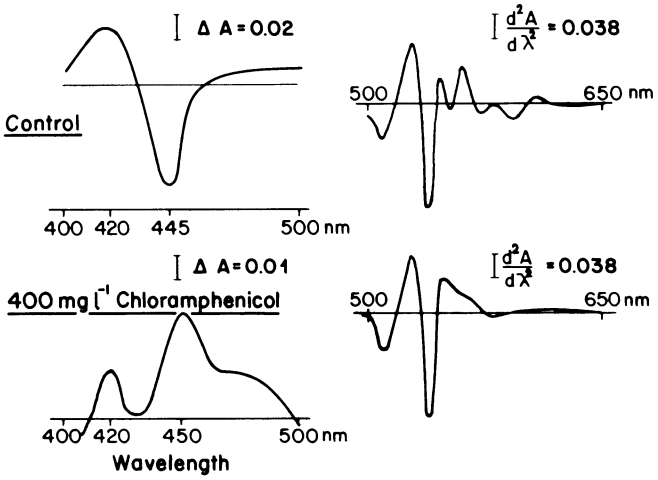

FIG. 5. Reduced CO difference spectrum (left) and second derivative of reduced minus oxidized spectrum (right) of aerobically grown cells of *S. uvarum* in the presence and absence of chloramphenicol. Chloramphenicol inhibits the synthesis of mitochondrial cytochromes *a* and *b* and therefore derepresses the formation of cytochrome P-450.

TABLE 3. Metabolic characteristics of *T. cutaneum*, *C. tropicalis*, and *S. uvarum*, the yeasts investigated for cytochrome P-450 occurrence

Characteristic	*T. cutaneum*	*C. tropicalis*	*S. uvarum*
Ethanol formation from glucose			
C limitation	−	−	+
O limitation	−	+	+
Growth of *n*-alkanes	−	+	−

C. tropicalis and *S. uvarum* grown under oxygen limitation was cytochrome P-450 detected.

To remove the interference from mitochondrial cytochromes, the cytochrome P-450-bearing fraction, the microsomes, was isolated by the method of Käppeli et al. (54) and the cytochrome P-450 content was determined (Table 4). All yeasts contained cytochrome P-450, with the concentration varying considerably with the cultivation conditions. Generally, oxygen limitation led to a derepression of cytochrome P-450 biosynthesis, independent of the yeast strain and substrate used. The low concentration of cytochrome P-450 in the microsomal fraction of *T. cutaneum*, a purely respiratory yeast, is remarkable. Metabolic characteristics seem to influence cellular cytochrome P-450 content. The results indicated, however, that it is probably generally present in yeasts.

Other important data on the distribution of cytochrome P-450 in yeasts resulted from studies by Kärenlampi et al. (57). The authors scanned various yeast species, many of which were found to produce cytochrome P-450 in 5% glucose-containing growth medium. Measurable levels of enzyme were found in strains of *Brettanomyces anomalus*, *Hansenula anomala*, *S. bayanus*, and *Torulopsis glabrata*, and especially in *S. italicus*.

Kärenlampi et al. (58) also investigated the induction of cytochrome P-450 in yeasts by chemicals known to have that effect for hepatic cytochrome P-450. Only 2 of 14 chemicals tested gave rise to a higher cytochrome P-450 content in yeasts. An average increase of 30% was noticed, with the individual values ranging from 20 to >100%. The question remains whether a specific induction occurred or whether

TABLE 4. Microsomal cytochrome P-450 content of different yeasts grown under different conditions[a]

Yeast species	Cytochrome P-450 content (pmol [mg of protein]$^{-1}$)
T. cutaneum	
Glucose, C limited	25
Glucose, O limited	70
C. tropicalis	
Glucose, C limited	40
Glucose, O limited	190
Alkane, C limited	210
Alkane, O limited	700
S. uvarum	
Glucose, C limited	35
Glucose, O limited	330

[a] Data are from references 66, 88, and 91.

the increase in cytochrome P-450 content was due to a secondary effect. The tested chemicals certainly affected growth and metabolism of the yeasts, which may lead to conditions favoring cytochrome P-450 biosynthesis as, for example, a shift to more pronounced fermentative metabolism. The same comment is valid for the results of Wiseman and Lim (102), who reported the induction of a cytochrome P-450 when the cells grew in the presence of phenobarbital. In the case of induction experiments, merely recording cellular cytochrome P-450 content is not adequate. Other physiological parameters need to be monitored (e.g., growth, ethanol production) to determine properly the specificity of added inducers.

Physiological Functions of Cytochrome P-450 in Yeasts

Since the detection of cytochrome P-450 in alkane-assimilating yeasts, it was postulated that the enzyme was involved in the terminal hydroxylation of *n*-alkanes. This reaction is generally accepted now to be the first step of the degradative pathway in alkane-utilizing microorganisms (31, 35, 64, 95). Available data from in vitro assays suggest that this is the physiological role of the alkane-induced cytochrome P-450 system in yeast cells (87, 88). However, up to now the in vitro turnover rates of the systems are much lower than would be required to cope with the in vivo substrate uptake rates. The in vitro activities are between 1 and 5 pmol of product formed (pmol of cytochrome P-450)$^{-1}$ min^{-1}. From a medium substrate uptake rate of 1 mmol g^{-1} h^{-1} and a medium cytochrome P-450 content of 40 pmol (mg of dry cell weight)$^{-1}$, a turnover number of 420 pmol (pmol of cytochrome P-450)$^{-1}$ min^{-1} is calculated, which is substantially higher than the in vitro activity. The low in vitro activity probably results from the poor mass transfer in the assay due to the insolubility of the substrate.

The biological function of cytochrome P-450 from *S. cerevisiae* has remained unclear for a long time. Alexander et al. (3) assumed its involvement in the late stages of ergosterol biosynthesis. This hypothesis was confirmed by Ohba et al. (84), who showed that the oxygen- and NADPH-dependent conversion of lanosterol to 4,4-dimethylzymosterol is inhibited by cytochrome P-450 antibodies. Shortly after that, the oxidative removal of the 14 α-methyl group of lanosterol by a reconstituted system of cytochrome P-450 and NADPH-cytochrome P-450 reductase was demonstrated (8).

Hata and co-workers (39) described an additional cytochrome P-450 reaction in yeasts, the Δ^{22}-desaturation of ergosta-5,7-dien-3β-ol. By using mutant strains which lacked the 14 α-demethylase, Δ^{22}-desaturation was still possible, which indicates that two distinct cytochrome P-450 species may be involved in ergosterol biosynthesis.

If the data on induction of cytochrome P-450 in *S. cerevisiae* (see preceding section) are considered in the context of the physiological function, it is difficult to establish a relation between increased cytochrome P-450 formation under oxygen-limited conditions and ergosterol biosynthesis. Considering this activity as terminal oxidase within an enzyme system which catalyzes special hydroxylation and demethylation reactions or the desaturation of fatty acids, a relation to ethanol formation in the cells may exist. It was indicated that fermentative glucose metabolism is required to get derepression of cytochrome P-450 biosynthesis (Fig. 3). The increased exposure of the cells to ethanol could require an adapted composition of cellular membranes which may involve an increased incorporation of ergosterol. This would explain the higher cytochrome P-450 content of cells subjected to strongly fermentative growth conditions. The relatively low cytochrome P-450 content of the strictly respiratory yeast *T. cutaneum* (Table 4) supports this hypothesis. It is further substantiated by investigations of Del Carratore et al. (27), Blatiak et al. (16), and Morita and Mifuchi (76). An increased cellular cytochrome P-450 content was found by these authors in the presence of ethanol. However, no data on lipid composition of cells exposed to ethanol are currently available.

Besides the involvement of cytochrome P-450 in lipid biosynthesis, another possible function should be considered. Cytochrome P-450 may be involved in an alternative regeneration of reducing equivalents known as cyanide-insensitive respiration (1, 2). Mitochondrial respiration, which creates energy, represents the preferred system as long as sufficient oxygen is available. When oxygen becomes limited the alternative pathway involving cytochrome P-450 becomes effective. This pathway would not produce energy but would regenerate NADH and especially NADPH. Hitherto no conclusive data on the participation of cytochrome P-450 in the cyanide-insensitive respiration have been available, but its role as NAD(P)H oxidase should be considered as a possible function in the metabolism of the cells.

ISOLATION AND PROPERTIES OF YEAST CYTOCHROMES P-450

Since the discovery of cytochrome P-450 in yeasts the comparison of the system with that of mammalian liver has constituted a substantial part of the research. The prospects of potential applications in a wide range of oxidation reactions as indicated by the hepatic cytochrome P-450 enzymes have stimulated work with the microbial systems. If cytochrome P-450 should ever be applied in replacing chemical hydroxylations it would be required in large amounts. Microbial cytochrome P-450 could therefore be a possible source. This requires that appropriate methods for production and isolation be available.

Cytochrome P-450 systems are relatively unstable and the small-scale laboratory preparations are inappropriate for scaling-up. Therefore, an account of the current state of isolation procedures and of the properties of yeast cytochromes P-450 are given in the following sections.

Isolation

Production of cells with high cytochrome P-450 content. The previous section reviewed the conditions for cytochrome P-450 induction and derepression in alkane- and glucose-grown cells, respectively. When these conditions are applied in batch cultures, problems with the determination of the harvest time at maximum cellular cytochrome P-450 content is difficult. The optimum concentration is obtained in a relatively narrow time interval (16), and batch cultures are not highly reproducible.

We have therefore used a continuous-culture technique for the production of cytochrome P-450-containing yeasts (35, 66, 88, 98). In this cultivation system the influence of individual parameters can best be evaluated and the best conditions can be maintained independently of time. It follows that identical cell material is obtained for long periods of time. A further advantage is that the actual substrate concentration in the cultivation vessel is close to zero. In cultivations involving hydrocarbon substrate this is of great importance because cells adhere to emulsified sub-

strate droplets (55). Only cells from continuous culture can be handled properly without interference of residual hydrocarbons.

For subsequent isolation of cytochrome P-450, the quality of the starting material will mainly influence final yield and initial contamination of the cytochrome P-450 fraction.

Isolation of cytochrome P-450. After the detection of cytochrome P-450 in cell extracts of *S. cerevisiae* (68), it was first reported by Ishidate et al. (49) that this hemoprotein was bound to a particulate fraction, the microsomes.

For the isolation of microsomes, cell breakage is required. Yeast cells may be disrupted mechanically or by spheroplasting the cells enzymatically before a mild mechanical treatment or osmotic shock is used to release the cell content. The instability of cytochrome P-450 requires that attention be paid to the method of cell disruption. Besides enzymatic lysis of cells (54), pressure (French press; 29, 32, 105, 107) and disruption of the cells in a Vibromill (62) or a Dynomill (93) were used for preparing cell-free extracts. The latter method may cause a partial denaturation of cytochrome P-450 (Fig. 6). However, differences in the stability of cytochromes P-450 from different yeast strains are observed, and the method to be applied needs to be examined with the yeast used. The importance of the cell disruption method is manifested by the fact that Duppel et al. (29) found the components of the monoxygenase system, i.e., cytochrome P-450 and the NADPH-cytochrome P-450 reductase,

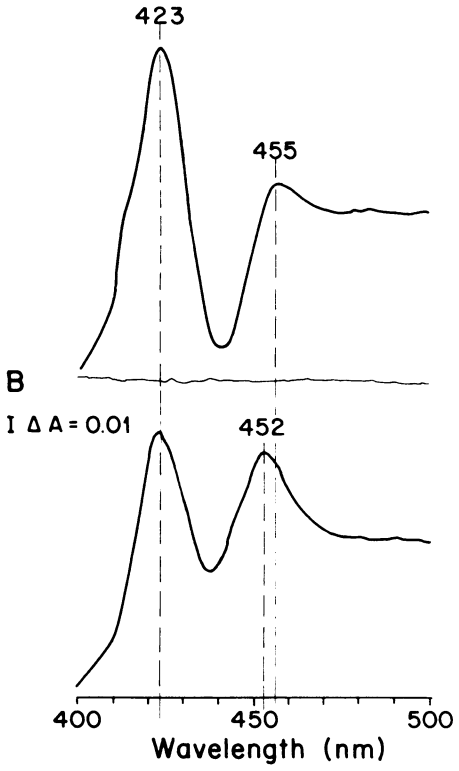

FIG. 6. Reduced CO difference spectra of crude extracts of *C. tropicalis* cells grown on *n*-alkanes which were disrupted mechanically (top) and enzymatically (bottom). More cytochrome P-450 is denatured to cytochrome P-250 after mechanical cell disruption. Cytochrome P-450 contents were 7.5 and 15.2 pmol (mg of protein)$^{-1}$, respectively. B, Base line.

TABLE 5. Cytochrome P-450 enrichment by a 100,000 × *g* centrifugation of a postmitochondrial supernatant and by $CaCl_2$ precipitation of the microsomal fraction from the same sample[a]

Sample	Cytochrome P-450	
	pmol ml^{-1}	pmol mg^{-1}
100,000 × *g* sediment	318.7	96.6
$CaCl_2$ microsomes	329.7	221.3

[a] The cytochrome P-450 content of the cell-free extract was 27.6 pmol (mg of protein)$^{-1}$ (54).

in the soluble fraction after disrupting the cells with a French pressure cell. This artifact was probably due to the disruption method used.

After preparation of the cell-free extract, the isolation of the microsomal fraction is further pursued by differential centrifugation (32, 62, 94, 107). First, a mitochondrial fraction is formed by one or two centrifugation steps. From the postmitochondrial extracts the microsomes are sedimented by high-speed centrifugation, usually 100,000 × *g*. Further purification of the microsomal fraction may be accomplished by density gradient centrifugation (32). Isolation of microsomes by differential centrifugation yields relatively low amounts of cytochrome P-450-bearing fraction, and it certainly is contaminated with other membrane fragments.

For isolation of the microsomal fraction from different yeasts, a method involving $CaCl_2$ aggregation was developed (54). This method lacks the disadvantages of centrifugal isolation techniques. It is relatively fast and suitable for scaling-up, and the resulting fraction is significantly more pure than that resulting from differential centrifugation (54). In $CaCl_2$-aggregated microsomes, cytochrome P-450 was spectrally clean. No mitochondrial markers (cytochrome oxidase) and only very low catalase activity (as cytoplasmic marker) were detected. The enrichment factor for cytochrome P-450 was significantly higher with the $CaCl_2$ aggregation method than with differential centrifugation when the same starting material was used (Table 5). In addition, the method was successfully used for different yeast species, i.e., *S. uvarum* (91), *C. tropicalis* grown on glucose or *n*-alkanes (88), and *T. cutaneum* (66).

From the microsomal fraction, cytochrome P-450 is solubilized by detergents. Duppel et al. (29) remain the only authors to report an apparently soluble form of cytochrome P-450. Probably their results represent an artifact originating from the method used for cell rupture (see above).

The difficulties connected with the isolation of membrane-bound enzymes include finding substances which are most active with respect to solubilization but which do not interfere with enzymatic activity. Detergents have proved to be most suitable for a careful solubilization of cytochrome P-450 from the microsomal fraction. First attempts to solubilize cytochrome P-450 by different types of detergents led, however, to the conversion to cytochrome P-420 (86). This disadvantage was overcome by utilizing glycerol as the stabilizing agent (45) and by the use of sodium deoxycholate, sodium cholate, or nonionic detergents for solubilization. Nowadays, mainly sodium cholate or 3-(<3-cholamidopropyl > dimethylamino) 1-propanesulfonate (42) is used for releasing proteins from membranes.

Solubilization of yeast cytochrome P-450 was effected by methods developed for the hepatic enzymes. In all reports so far, sodium cholate was used as the solubilizing agent for yeast cytochrome P-450 (62, 87, 104). No data are available

TABLE 6. Summary of published yeast cytochrome P-450 purification methods[a]

Method	Reference		
	87	62	104
Solubilization	Na-cholate	Na-cholate	Na-cholate
1. Fractionation		$(NH_4)_2SO_4$	$(NH_4)_2SO_4$
1. Separation	*N*-Amino-octyl–Sepharose 4B	*N*-Amino-octyl–Sepharose 4B	6-Aminohexyl–Sepharose 4B
2. Separation	Calcium phosphate gel	Hydroxylapatite-cellulose	Hydroxylapatite
3. Separation		CM-Sephadex C-50	CM-Sepadex C-50
4. Separation		DEAE-Sephacel	
Specific content (nmol [mg of protein]$^{-1}$)	10.6	16–17.5	18

[a] Riege et al. (87) used *n*-alkane-grown *L. elongisporus*; King et al. (62) and Yoshida and Aoyama (104) isolated cytochrome P-450 from *S. cerevisiae* species. CM, Carboxymethyl; DEAE, diethylaminoethyl.

on the efficiency of solubilizing methods involving yeast cytochrome P-450.

For further enrichment of cytochrome P-450, ammonium sulfate fractionation and liquid chromatographic methods were used. A summary reveals that the methods used for the three yeast cytochromes P-450 purified so far do not differ very much (Table 6). Again, they are derived from methods developed for hepatic cytochrome P-450 (48). A notable alteration is the use of 6-aminohexyl–Sepharose 4B, instead of 8-amino-*n*-octyl–Sepharose 4B, for the hydrophobic chromatography by Yoshida and Aoyama (104). Other minor differences concern the ionic strength of buffers. Although the content of cytochrome P-450 in yeast cells is much lower than in liver microsomes, purification is obviously possible. The specific content of the three reported preparations varied and consequently probably their degree of purity also varied. Differences may also arise, however, from interactions of residual detergent with the protein determination or from the presence of apoprotein devoid of the heme group, which therefore is not accounted for in the spectrophotometric determination of cytochrome P-450 content.

Properties

Molecular weight. The molecular weights (determined by sodium dodecyl sulfate gel electrophoresis) of the monomeric forms of cytochromes P-450 were 53,000 for that from *Lodderomyces elongisporus* (87) and 55,500 and 58,000, respectively, for those isolated with *S. cerevisiae* (62, 104). With respect to molecular weight, yeast cytochromes P-450 are, therefore, similar to mammalian ones, as discussed in the original reports (62, 87, 104).

TABLE 7. Spectral parameters of purified yeast cytochrome P-450

State	λ_{max} in reference:		
	87	14	105
Oxidized	568	578	575
	536	565	540
	417	417	418
			360
Reduced	550	578	550
	414	565	412
		418	
Reduced CO complex	550	560	555
	447	446	447
		562	
Reduced CO difference	447	447–448	448

[a] Sources of cytochromes P-450 were *L. elongisporus* (87) and two strains of *S. cerevisiae* (14, 105).

Spectral properties. The most distinctive spectral property of cytochromes P-450 is their absorbance at around 450 nm in the reduced CO difference spectrum. Purified yeast cytochromes P-450 exhibited a maximum at 447 to 448 nm (Table 7). An additional characteristic of the cytochrome P-450 hemoprotein family is an abosrption at around 550 nm for both the reduced form and the reduced CO complex, which is also obvious in the purified yeast preparations (Table 7). The Soret absorption at 417 nm of the oxidized states indicates that the oxidized form is in a low spin state. Together with the Soret peak at 447 nm of the reduced CO complex, it more precisely classifies the yeast cytochromes P-450 as members of the "P-448" group. Dissimilarities to low spin cytochromes P-450 of mammalian origin were, however, reported by Yoshida and Aoyama (104). Some features of the circular dichroism spectra of *S. cerevisiae* cytochrome P-450 suggested certain pecularities in the heme environment, but nothing is known as yet.

Spectral ligand binding. Upon interaction with various compounds, oxidized cytochromes P-450 exhibit spectral changes known as "substrate-induced difference spectra" (86, 92). Substrates of the mixed-function oxidase induce a type I spectral change (Fig. 7). Other substances that interfere with the heme group of cytochrome P-450 cause a type II spectral change (Fig. 7). The latter is characteristic, for example, of cytochrome P-450 inhibitors.

Müller et al. (78) added several compounds to the oxidized form of purified cytochrome P-450 from *L. elongisporus*. Only hexadecane, the growth substrate of the cells, gave a type I spectral change. All other compounds tested (aminopyrine, benzphetamine, imidazole, aniline, etc.) exhibited type II or reversed type I spectral changes.

Similarly, Yoshida and Aoyama (104) observed a type I spectral change with lanosterol, thought to be the natural substrate of cytochrome P-450 from *S. cerevisiae*. Other compounds such as aniline, metyrapone, imidazole, and pyridine showed a type II spectral change. Noteworthy is the fact that these authors did not observe a type I spectral change with benzo(a)pyrene, which is in contrast with data reported by King et al. (62). The latter authors also observed type I binding spectra with lanosterol, ethylmorphine, dimethylnitrosamine, sodium phenobarbitone, and perhydrofluorene. Type II spectral changes resulted with imidazole, aniline, and benzphetamine.

Other important properties. The heme group of the cytochrome P-450 from *S. cerevisiae* was determined as protoporphyrin IX by Yoshida and Kumaoka (106). Yeast

cytochrome P-450 bound with ethyl isocyanide in both the oxidized and reduced states (104, 106). The ethyl isocyanide complex of the reduced cytochrome showed two Soret peaks at 430 and 455 nm, and the relative intensity of these peaks was dependent upon pH of the medium as is the case with hepatic cytochrome P-450 (38). This finding indicates that the yeast cytochrome also exists in two interconvertible forms (called the 430 and 455 forms) in the reduced state.

As in the case of hepatic cytochrome P-450, yeast cytochrome can be denatured to cytochrome P-420. Suitable denaturating agents include several organic solvents, high concentrations of detergents, high concentrations of chaotropic anions, and mercurials such as *p*-chloromercuribenzoate and $HgCl_2$ (104). The yeast cytochrome is considerably more resistant to treatment with nonionic detergents than is the hepatic cytochrome P-450.

Stability

Stability of yeast cytochromes P-450 is rarely discussed, although their decay poses serious problems in isolation and application. As mentioned above (Fig. 6), the cell disruption method may lead to denaturation. Other factors influencing stability are pH and ionic strength of buffer and temperature.

Generally, yeast cytochrome P-450 is more stable in buffers of higher ionic strength; e.g., its stability is increased in 100 mM Tris hydrochloride buffer as compared to a 10 mM

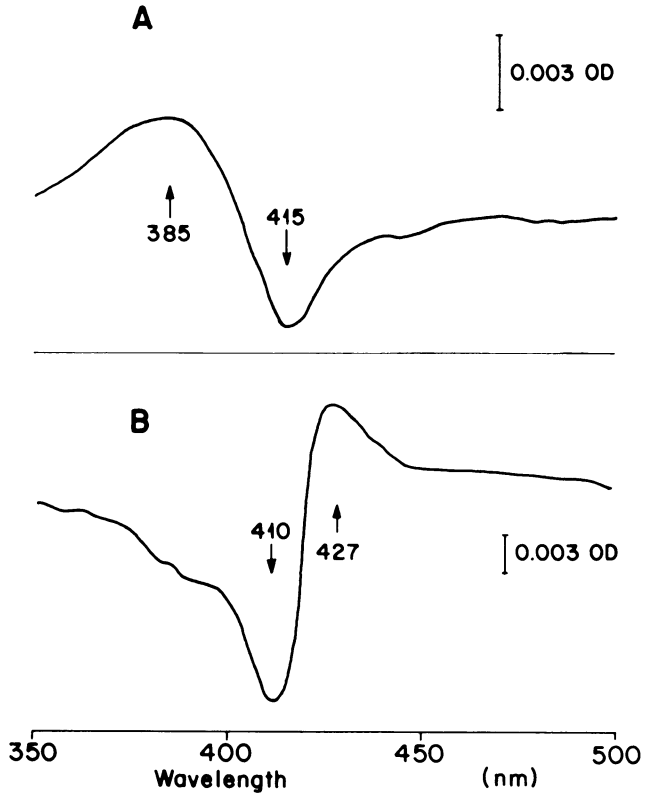

FIG. 7. Type I (A) and II (B) spectral changes caused by the addition of lanosterol and propiconazole, respectively, to the microsomal fraction of glucose-grown *C. tropicalis*. Type I spectral changes exhibit an absorption maximum at about 385 nm and a minimum at about 415 nm. Type II spectral changes are characterized by an absorption maximum varying between 425 and 435 nm and a broad trough between 390 and 410 nm. OD, Optical density.

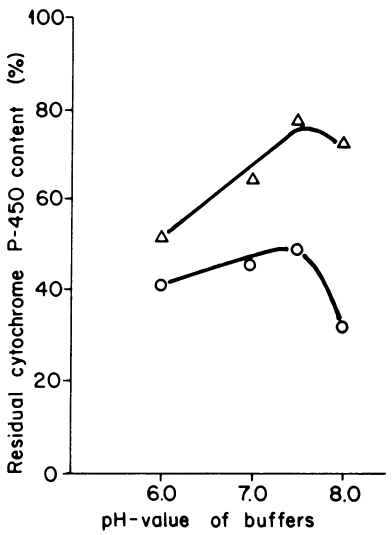

FIG. 8. Dependence of stability of cytochrome P-450 from *C. tropicalis* on pH of the buffer. The curves indicate residual cytochrome P-450 after 1 (△) and 2 (○) days of storage.

concentration. The optimum pH for stability lies between 7.0 and 7.5 (Fig. 8).

Temperature influences the stability of cytochromes P-450 significantly. When it was assessed in the reaction mixture of a monooxygenase assay with a microsomal fraction of *C. tropicalis* at 30°C, 20% was lost during an incubation period of 2 h. At 37°C, 50% of the cytochrome P-450 was denatured in 1 h (D. Sanglard and O. Käppeli, unpublished data). It follows that cytochrome P-450 reaction rates need to be analyzed carefully to avoid erroneous conclusions.

Low-temperature storage at −20°C also did not prevent long-term losses completely. Significant denaturing was observed after 4 months of storage (Sanglard and Käppeli, unpublished data). As a general rule it can be assumed that significant losses of cytochromes P-450 are encountered after periods of hours at 30°C, of days at 4°C, of months at −20°C, and of years at −80°C. Deviations in the decay rate may result from the fraction used for stability testing. Purified cytochrome P-450 is generally more stable than that retained in the microsomal fraction. Furthermore, differences may also arise from the source of cytochrome P-450. It was found, for example, that cytochrome P-450 from *C. tropicalis* was less stable than that from *S. uvarum* (Sanglard and Käppeli, unpublished data). The data reported here were all recorded in buffers containing the known cytochrome P-450 stabilizers dithiothreitol and glycerol (4, 44). It follows that even in the presence of stabilizers cytochrome P-450 denaturation is not prevented completely and has to be considered in any dealing with this hemoprotein.

HETEROGENEITY OF YEAST CYTOCHROME P-450 MONOOXYGENASES

The specificity of cytochrome P-450 monooxygenases is determined by the terminal oxidase. The most distinct properties of yeast cytochromes P-450, indicating variations in the particular preparation, were the molecular weight and the spectral ligand-binding studies, as reviewed in the previous section. These differences indicate specificity in the reactions catalyzed by the monooxygenase systems. To assess the catalytic properties of yeast monooxygenases,

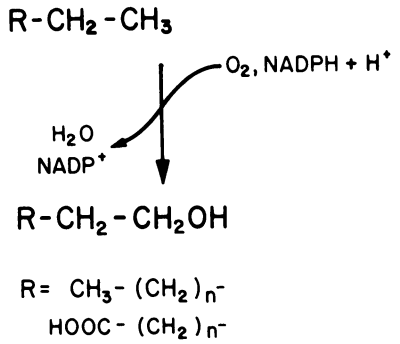

FIG. 9. Main catalytic activity of cytochrome P-450$_{aOH}$ originating from hydrocarbon-grown yeasts.

studies with microsomal preparations and reconstituted systems were carried out.

Components of Yeast Cytochrome P-450 Monooxygenases

For the first time, the hydroxylating system from *C. tropicalis* was resolved by Duppel et al. (29). It consisted of three components: cytochrome P-450, NADPH-cytochrome P-450 reductase, and a thermostable lipid fraction. In reconstitution of the active enzyme system, yeast reductase and lipid were exchangeable with the respective fractions from rat liver microsomes. These results indicate a strong similarity between the monooxygenase from yeasts and that of the hepatic microsomal fraction with respect to structural organization and electron transport. Furthermore, the study supported the idea that the specificity of the monooxygenase is determined by the terminal oxidase, cytochrome P-450.

The reductase was subsequently purified from *L. elongisporus* (43). The reductase from *S. cerevisiae* was isolated, enriched to homogeneity, and finally characterized by Aoyama et al. (10). The enzyme has many properties in common with that from hepatic microsomes. King et al. (62) isolated the reductase from another *S. cerevisiae* strain and used it in studies with reconstituted yeast monooxygenase.

Types of Yeast Monooxygenases

The activity of monooxygenases originating from different yeasts was analyzed, using microsomal fractions or reconstituted systems. Two distinct activities were distinguished: monooxygenase-catalyzing aliphatic hydroxylation and that being involved in the 14α-demethylation of lanosterol.

Aliphatic hydroxylation system. Aliphatic hydroxylation of long-chain fatty acids (ω-hydroxylation) and alkanes (Fig. 9). are the reactions proved and generally accepted for the cytochrome P-450 monooxygenase systems from hydrocarbon-grown yeasts (15, 29, 32, 67, 87, 88, 91). When the activity is measured with microsomes and *n*-alkanes, the corresponding fatty acid is the reaction product. The microsomal fractions also contain high activities of alcohol and aldehyde dehdrogenases. The rate-limiting step seems to be the primary hydroxylation. In assays in which reconstituted monooxygenases are used, the product is always the corresponding hydroxylation derivative, as is the case whenever fatty acids are used as substrates (15, 29, 88).

Furthermore, Lebeault et al. (67) and Duppel et al. (29) detected the N-demethylation of aminopyrine, benzphetamine, and ethylmorphine with a partially purified preparation from alkane-grown *C. tropicalis*. It was concluded that this monooxygenase hydroxylates a variety of fatty acids and drugs (29). These results have to be regarded as preliminary. The activities were determined by measuring the release of formaldehyde, which is a by-product of the monooxygenase-catalyzed demethylation reactions. However, the formation of the main products, i.e., the converted educts, was not shown. Furthermore, the conversion rates were low and the actual involvement of cytochrome P-450 was not conclusively proved (as, e.g., by specific inhibitory studies). In fact, N-demethylation of benzphetamine by microsomes of *L. elongisporus* was not inhibited by carbon monoxide (77), a specific monooxygenase inhibitor.

Since cytochrome P-450 is the specificity-determining component of the monooxygenase, and aliphatic hydroxylation is the characteristic reaction catalyzed by the microsomal fraction originating from different hydrocarbon-grown yeasts, it is suggested that this terminal oxidase be designated cytochrome P-450$_{aOH}$ (subscript for aliphatic hydroxylation). This nomenclature defines the cytochrome P-450 with respect to origin and main catalytic activity and is also in accordance with that introduced by Yoshida and Aoyama (104) for the 14α-demethylase of lanosterol (see below).

14-α-Demethylation system. The catalytic activity of the monooxygenase from *S. cerevisiae* differs markedly from that of alkane-grown yeasts. Sanglard et al. (88) were not able to find any aliphatic hydroxylation activity with the monooxygenase of glucose-grown cells of *C. tropicalis*, whereas in alkane-grown ones the system was present. Obviously, biosynthesis of the two forms was influenced by cultivation conditions. More recent data (53) indicate that the monooxygenase of glucose-grown *C. tropicalis* very much resembles that of *S. cerevisiae*, with 14α-demethylation of lanosterol being the characteristic activity which was established and experimentally verified by Aoyama and co-workers (8, 10–12). A mechanism for the involvement of cytochrome P-450 in sterol synthesis was postulated (Fig. 10).

For the cytochrome P-450$_{14DM}$ monooxygenase, catalytic activities other than the 14α-demethylation of lanosterol were reported. The benzo(a)pyrene hydroxylation has been studied most extensively (14, 62). This activity, however, was not confirmed by Aoyama et al. (11). Neither spectral ligand binding nor benzo(a)pyrene hydroxylation was observed by these authors. Since both groups used members of the same yeast species (*S. cerevisiae*) as a source of monooxygenase, this indicates that multiple forms of cytochrome P-450 might exist.

With a cytochrome P-450-enriched microsomal fraction of *S. cerevisiae* var. *uvarum*, demethylation of aminoyrine, *p*-nitroanisole, and caffeine were also reported (91). But again these results are preliminary since the activity was measured by formaldehyde determination, which may be artefactual.

The cytochrome P-450 from *S. cerevisiae* was designated cytochrome P-450$_{14DM}$ by Yoshida and Aoyama (104). This nomenclature is based on the characteristic activity of the system, the 14α-demethylation of lanosterol. The data available on yeast cytochrome P-450 clearly indicate that the two cytochrome P-450 forms discussed here are catalytically different, and therefore a distinction (by using a subscript) is reasonable. This is supported by an immunological investigation by Kärgel et al. (59). A very weak cross-reaction of antiserum against *C. maltosa* cytochrome P-450$_{aOH}$ with microsomes of *S. cerevisiae* was observed in a competitive radioimmunoassay. In the Ouchterlony test no interaction

was found, which indicates that the two forms are only slightly related, if at all.

Other Yeast Monooxygenases

Considering the verified activities of yeast monooxygenases, a classification as narrow-specificity oxidases is adequate. There seem to be few overlapping activities of the cytochrome P-450$_{\omega OH}$ and P-450$_{14DM}$ systems. However, the occurrence of two distinct monooxygenases also signifies that different forms may be produced by yeasts. Should it be possible to induce specific cytochrome P-450 forms by changing the yeast strain or varying the cultivation conditions, it would be of great interest for both basic research and practical applications. In liver there are always several cytochrome P-450 enzymes present, making analytical work more difficult.

It is remarkable that in *C. tropicalis* two distinct forms of cytochrome P-450 are formed dependent upon the cultivation conditions: cytochrome P-450$_{\omega OH}$ when the cells grow on hydrocarbon, and (most probably) cytochrome P-450$_{14DM}$ when cells grow on glucose (53). Possibly other cytochromes P-450 may be induced under appropriate conditions. When biphenyl hydroxylation was assessed with whole cells of *C. tropicalis* 4-OH-biphenyl was the only product. It is probable that a distinct cytochrome P-450 form is induced by biphenyl (53). Furthermore, analogous experiments with *T. cutaneum* yielded 2-, 3- and 4-OH-biphenyl as reaction products, which indicates that possibly a cytochrome P-450 of other specificity may be induced in *T. cutaneum* than in *C. tropicalis* by the same inducer, biphenyl (53). These rather preliminary results signify that specific cytochromes P-450 may be obtained by changing yeast strains and induction conditions.

Cytochrome P-450 Inhibitors

For the characterization of oxidation reactions, inhibitory studies contribute to the determination of the nature of the system involved. Carbon monoxide inhibition is a characteristic property of cytochrome P-450-dependent activities. Carbon monoxide interacts with the heme part of cytochrome P-450 and thus inhibits substrate interaction and transformation. It is, therefore, often used as an indicator of cytochrome P-450 monooxygenase activity.

Other cytochrome P-450 inhibitors have recently become of great interest, since many of them are used in antifungal therapy in both human and veterinary medicine. In general, antifungal agents interfere with sterol synthesis and belong to the group of azole-containing compounds, e.g., imidazole and triazole derivatives and nonazole nitrogen heterocycles (99). For a substantial number of these agents, the activity is based on the interference with the microsomal, cytochrome P-450-dependent lanosterol 14α-demethylase system. In his review (99) Van den Bossche lists close to 20 imidazole and triazole derivatives which act as cytochrome P-450 inhibitors. Representatives of this group are ketoconazole, miconacole, etaconazole, and propiconazole. The application of these compounds in therapy is based on the selective toxicity they exhibit on cytochromes P-450 of different origins. The cytochromes P-450 of rat liver microsomes are ca. 1,000-fold less sensitive to, for example, itraconazole, ketokonazole, and miconazole than are those of *S. cerevisiae* microsomes (99).

Buthiobate, a nonazole derivative, was also identified as a potent inhibitor of cytochrome P-450$_{14DM}$ (9). It bound stoichiometrically and induced a type II spectral change. Buthiobate and other inhibitors may be of great value in characterizing cytochromes P-450 of different origins.

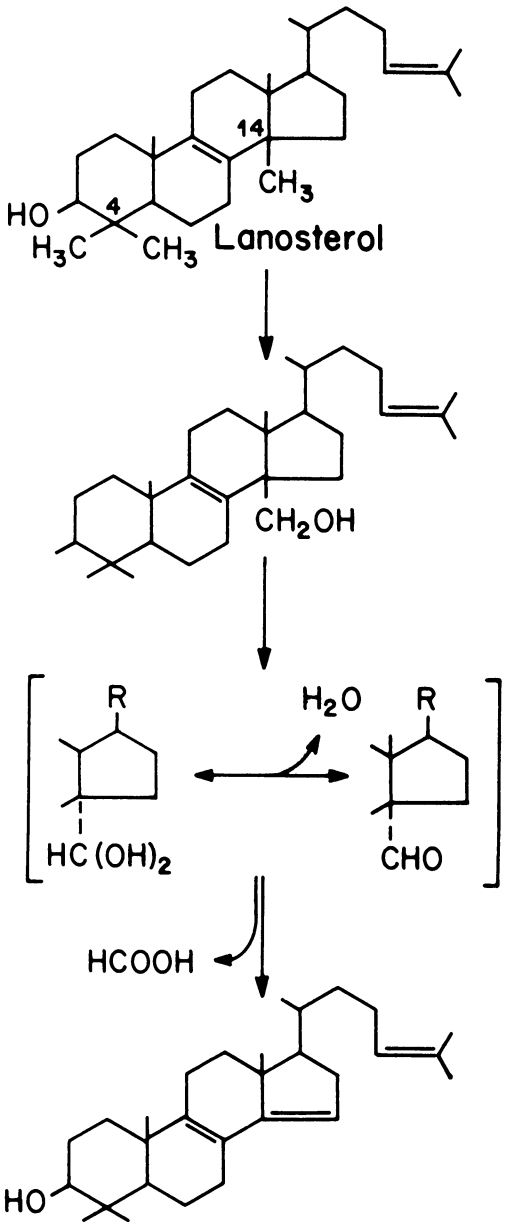

FIG. 10. Main catalytic activity of cytochrome P-450$_{14DM}$ originating from glucose-grown yeasts (11).

UTILITY OF YEASTS IN CYTOCHROME P-450-RELATED ACTIVITIES

Having reviewed the knowledge existing on yeast cytochromes P-450, the question arises as to the significance of these enzymes. Where and how may yeast become an important factor in areas of cytochrome P-450 research and applications? These aspects are discussed below.

Yeasts as Hosts of Cloned Cytochrome P-450 Genes

Cytochromes P-450 are expressed at a relatively low level in yeasts. The concentration in the microsomal fraction

amounts to 1 to 10% of that in hepatic microsomes. Highest concentrations are noticed with the inducible cytochrome P-450$_{aOH}$. Low levels are present in glucose-grown cells under aerobic conditions. For practical applications, expression needs to be enhanced. Since possibilities offered by optimizing the cultivation conditions have probably been exhausted, genetic methods need to be applied. The most promising approach is to clone the cytochrome P-450 genes and put them under control of a more potent promoter, as, for example, the alcohol dehydrogenase I promoter (7). In this way different yeast cytochromes P-450 could be expressed at higher levels which would probably help to improve the activities of the different monooxygenases in yeasts.

So far, no reports on the cloning of yeast cytochrome P-450 genes have been published. There exists a technical report by Loper et al. (69) in which the engineering of cytochrome P-450 genes in yeast for biodegradation is announced. This indicates that corresponding work is in progress.

The suitability of yeasts as hosts for mammalian cytochromes P-450 has been demonstrated. Oeda et al. (83) successfully expressed the major form of 3-methylcholanthrene-inducible rat liver cytochrome P-450 (P-450MC) in *S. cerevisiae*. An expression plasmid for the cloned cytochrome P-450MC complementary deoxyribonucleic acid was constructed under the control of the yeast alcohol dehydrogenase I promoter. The expression, subcellular location, and aryl hydrocarbon hydroxylase activity towards benzo (a)pyrene of cytochrome P-450MC synthesized in yeast, were analyzed (83). Most of the cytochrome P-450MC was found in the yeast microsomes as part of a functional electron transport chain which exhibited benzo(a)pyrene hydroxylase activity.

This report of expression of functional mammalian cytochrome P-450 in yeasts is of great significance. It indicates that electron transport to foreign cytochrome P-450 can be effected with yeast NADPH-cytochrome P-450 reductase. Thus, it can be expected that electron transport and consequently monooxygenase activity will be achieved with other mammalian cytochromes P-450 expressed in yeasts. This opens the possibility of insertion into yeasts of genes of distinct forms of mammalian cytochromes P-450 with particular catalytic activities. Therefore, it should eventually be possible to use yeasts for performing any of the many reactions mediated by cytochromes P-450, whatever their origin. This is a highly attractive prospect for application of cytochromes P-450.

Use of Yeasts in Basic Research on Cytochrome P-450

As outlined in the Introduction, many aspects of the reaction mechanism of cytochrome P-450 have not yet been elucidated satisfactorily. Research involving the hepatic system is difficult because there is not a single form of cytochrome P-450 expressed. Therefore, complexities arise from the presence of multiple forms. In yeast cells, under appropriate conditions, only one form of cytochrome P-450 is expressed. Thus, it is more accessible to investigations of the catalytic cycle and of the physical and mechanistic interactions of the individual components of the electron transport chain located in the microsomal membrane.

That foreign cytochrome P-450 genes can be expressed in yeasts as a functional entity will help to elucidate the basis of cytochrome P-450 specificity. When the sequences of individual cytochromes P-450 are known, the transformation of one form into another form should become possible by specific sequence alterations. By this means the region of the protein responsible for its specificity can be determined, and thus the evolution of cytochrome P-450 isozymes may become manifest.

Use of Yeasts in Mutagenicity Testing

The Ames test (6) is currently the most widely used short-term test for screening environmental mutagens and carcinogens. In the test the potentially mutagenic or carcinogenic compounds are treated with liver homogenates (the S9 fraction) prepared from rats which were pretreated with Aroclor to induce cytochrome P-450. These liver homogenates are able to metabolize most promutagens to their active, mutagenic forms. Mutagenicity is subsequently tested in *Salmonella typhimurium*. The success of the system is based on the fact that many carcinogens are also mutagens (74).

The in vitro activation of promutagens has the disadvantage that the active metabolite is generated outside the indicator cell. Transport is necessary through the cell envelope and the cytoplasm before interaction with deoxyribonucleic acid is possible. Therefore, if the active metabolite is highly labile or reactive, this interaction may not occur. Other disadvantages may arise from secondary metabolism or binding of the active metabolite to proteins within the S9 faction. These problems may be circumvented if the indicator cells are capable of metabolizing procarcinogens. Yeasts have been evaluated for such a purpose and activation of promutagens was actually found to take place (18, 60). A correlation with cytochrome P-450 content of the cells was established in that high cellular cytochrome P-450 content yielded better activation. These initial observations were further pursued (17, 19, 20, 21, 26), and there are indications that yeasts may eventually be used in mutagenicity testing.

There remain, however, several problems that need to be solved. The main question is, of course, whether yeast cytochrome P-450 systems yield the same metabolites as the hepatic S9 fraction. As discussed above, yeast cytochromes P-450 described so far are of narrow specificity. Further, the research performed does not conclusively demonstrate that activation exclusively proceeds via the cytochrome P-450 system. On the other hand, the possibility of cloning different mammalian cytochrome P-450 genes offers the use of constructed yeast strains that contain a specific activation system. This is a very promising aspect which should promote yeasts as short-term mutagenicity test organisms.

CONCLUSIONS

Cytochrome P-450 is the terminal oxidase of a monooxygenase which is widely distributed in nature and serves essential functions. Most prominent is its involvement in the metabolism of many xenobiotics in liver. The versatile catalytic activity of the system is determined by the occurrence of multiple forms of cytochrome P-450.

In yeasts two forms of cytochrome P-450 have been isolated so far, P-450$_{aOH}$ and P-450$_{14DM}$, the main catalytic activities of which are aliphatic hydroxylation of alkanes and fatty acids and 14α-demethylation of lanosterol, respectively. The data available indicate that they belong to narrow-specificity cytochromes P-450. Nevertheless, there are indications that multiple forms of cytochrome P-450 may be produced in yeasts by varying yeast strains and cultivation (induction) conditions.

There are two main reasons why yeasts may become predominant in substituting for hepatic cytochrome P-450 systems. (i) In contrast with bacterial monooxygenases (e.g., that of *P. putida* [36]), the arrangement of the microsomal electron transport chain in yeasts is equivalent to that of liver microsomes. Consequently, for changing the activity of the monooxygenase, cytochrome P-450 is the only part that has to be adapted. (ii) Yeasts are widely used microorganisms. Both genetic and cultivation methods are well established.

The knowledge of yeast biology should make it possible in the near future to enhance expression of yeast cytochrome P-450 or to express foreign cytochrome P-450 genes in this organism. By this means, the potential of cytochrome P-450 monooxygenases may be exploited more fully.

ACKNOWLEDGMENTS

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