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THE ROLES OF MICROSOMAL ELECTRON TRANSPORT SYSTEM IN LANOSTEROL DEMETHYLATION IN YEAST

YURI AOYAMA

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ABBREVIATIONS AND TRIVIAL NAMES OF COMPOUNDS

ABBREVIATIONS

$P\text{-}450_{14DM}$:	a cytochrome P-450 of yeast catalyzing lanosterol 14α-demethylation
DLPC:	1,2-dilauroyl L-α-phosphatidylcholine
GC-MS:	gas chromatography-mass spectrometry
GLC:	gas-liquid chromatography
TLC:	thin-layer chromatography

TRIVIAL NAMES OF COMPOUNDS

Ergosterol:	ergosta-5,7,22-trien-3β-ol
Lanosterol:	4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol
Zymosterol:	5α-cholesta-8,24-dien-3β-ol
Buthiobate:	S-butyl S'-p-tert-butylbenzyl N-3-pyridyldithiocarbonimidate
Metyrapone:	2,3-bis(3-pyridyl)-2-methylpropan-3-one
SKF-525A:	2-diethylaminoethyl 2-dipenylpropylacetate

CHAPTER I

INTRODUCTION

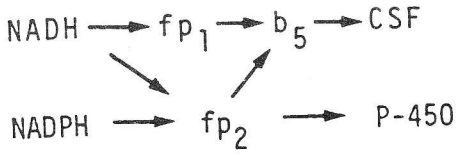
Brief History of Studies on Yeast Microsomal Electron-Transport System: The presence of non-mitochondrial heme proteins in anaerobically grown Saccharomyces cerevisiae was first reported by Euler and Fink (1) in 1927. About 30 years later, Lindenmayer and Smith (2) reinvestigated these heme proteins precisely and found the presence of cytochrome P-450- and P-420-like CO-binding pigments in addition to the non-mitochondrial heme proteins called cytochromes a_1 and b_1 (3,4) in anaerobically grown S. cerevisiae. Soon after this finding, heme proteins of anaerobically-grown yeast were analyzed in cell-free system by Ishidate et al. (5,6), and it was reported (5) that a cytochrome P-450-like CO-binding pigment and cytochrome b_1 were bound to a particulate fraction and were reduced by NAD(P)H.

In recent years, extensive studies on the non-mitochondrial heme proteins of semi-anaerobically grown yeast have been performed by Yoshida and his associates (7-18). They investigated these heme proteins as the postulated components of yeast microsomal electron-transport system and obtained the following observations. 1) A b-type heme protein formerly called cytochrome b_1 was purified, characterized and identified as cytochrome b_5 (7,10,18). 2) A CO-binding pigment showing its Soret peak at about 450 nm was purified, characterized and identified as cytochrome P-450 (8,11,16). 3) Two flavoproteins, NADH-cytochrome b_5 reductase (14) and NADPH-cytochrome P-450 reductase (13,15,17), were isolated and their similaritis with the hepatic microsomal counterparts were demonstrated. 4) Cytochrome b_5 and cytochrome P-450 were shown to be reduced by either NADPH or NADH when they were in microsomes (9). 5) The cytochrome b_5-containing system catalyzed the oxidative desaturation

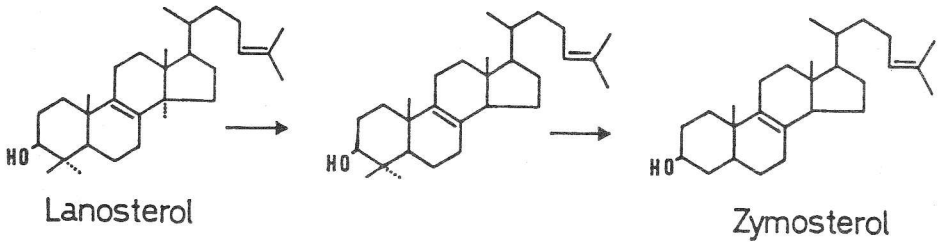
of palmitoyl-CoA, and this system contained a cyanide-sensitive factor as the terminal desaturase (12). In 1973, Duppel et al. (19) reported that cytochrome P-450 of Candida tropicalis, a petroleum assimilating yeast, grown aerobically on tetradecane could catalyze hydroxylation of hydrocarbons, fatty acids and drugs. As a result of these lines of investigations, it is now clear that yeast microsomes contain an electron-transport system (Scheme I-1) comparable to the hepatic microsomal one not only in its construction but also in its functions. However, cytochrome P-450 of S. cerevisiae could not catalyze the hydroxylation of hydrocarbon, fatty acids, etc. and its function has not yet been clarified.

Sterol Metabolism Occurring in Yeast Microsomes: In yeast cells, sterols are synthesized from acetate via mevalonate and squalene as the important intermediates (20). Lanosterol, the first sterol appearing in the biosynthetic pathway, is formed by cyclization of squalene (20) and then converted to ergo= sterol (20), the main sterol of yeast. It is now known that most of the reactions included in the conversion of lanosterol to ergosterol are catalyzed by enzymes localized in microsomal fraction (21,22). Among these reactions, three demethylations resulting in the conversion of lanosterol to zymosterol are observed commonly in microsomes of yeast and mammals and require NADPH and molecular oxygen (23) (Scheme I-2).

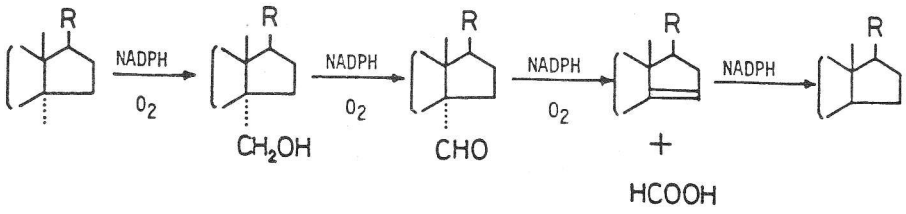
The initial step of these demethylations is the removal of the 14α-methyl group (C-32) of lanosterol (24). Chemical pathway of the 14α-demethylation has extensively been studied by Akhtar, Mitropoulos and their associates (25-33) and it has been reported that C-32 of lanosterol is removed as formic acid (26, 31-33) (Scheme I-3). In recent years, inhibitory effect of carbon monoxide was reported on the conversion of lanosterol to zymosterol by yeast cell-free system (28) as well as on the metabolism of lanosterol to cholesterol by mammalian microsomes (27). It was also found that carbon monoxide blocked



Scheme I-1. Microsomal electron transport system of yeast. fp_1; NADH-cytochrome $\underline{b}_5$ reductase, fp_2; NADPH-cytochrome P-450 reductase, $\underline{b}_5$; cytochrome $\underline{b}_5$, P-450; cytochrome P-450, CSF; cyanide-sensitive factor



Scheme I-2. Conversion of lanosterol to zymosterol catalyzed by microsomes of yeast and mammals.



Scheme I-3. Chemical pathway proposed for lanosterol 14α-demethylation.

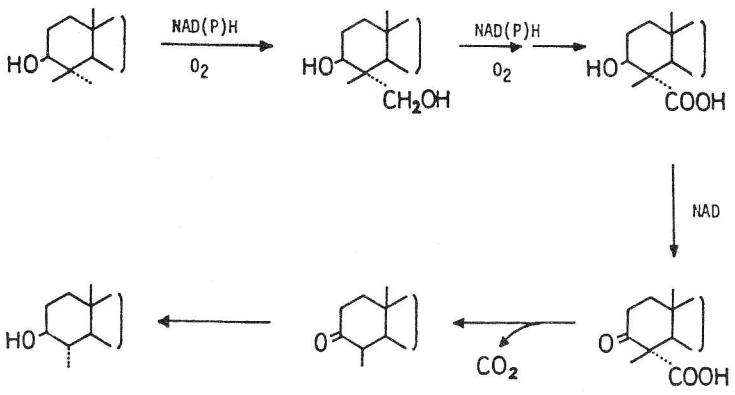
the removal of C-32 of 24,25-dihydrolanosterol as formic acid by rat liver microsomes and cell-free system of yeast (31). Based simply on these obser= vations, it was suggested that cytochrome P-450 contributes to the removal of C-32 of lanosterol both in yeast and mammals.

The two methyl groups (C-30 and C-31) of lanosterol are known to be removed as carbon dioxide (34). Mechanism of this demethylation by liver microsomes has been studied by Gaylor and coworkers (35-40) and the reaction has been considered to occur as shown in Scheme I-4. Although this demethylation required NADPH and molecular oxygen, the reaction occurring in liver micro= somes was inhibited not by carbon monoxide but by cyanide (36). Recently, it was reported that liberation of carbon dioxide from the α-methyl group at C-4 of lanosterol by yeast microsomes was also inhibited by cyanide (41). These facts suggest a possibility that the 4-demethylation is catalyzed by a cyanide-sensitive enzyme both in yeast and mammals and cytochrome P-450 does not contribute to this reaction as in the case of fatty acyl-CoA desaturation (12,42-44).

Judging from these observations, it is likely that the conversion of lanosterol to zymosterol is catalyzed by the electron-transport system of microsomes both in yeast and mammals. However, there is no direct evidence indicating the contribution of microsomal electron-transport system to the lanosterol demethylations. In this study, the author examines the relation= ship between microsomal electron-transport system and lanosterol demethylations in yeast cells to confirm the roles of the electron-transport system in the lanosterol demethylations.

In recent years, a great number of molecular species of cytochrome P-450 have been isolated from various sources and they have been called variety of tentative names. So, in this paper, the term "cytochrome P-450" is used collectively and individual molecular species are called by tentative names

given by their authors. The yeast cytochrome P-450 which is one of the subjects of this work catalyzes lanosterol 14α-demethylation as described below. So, the author used the tentative name "P-450$_{14DM}$" to distinguish this cytochrome P-450 species from others.



Scheme I-4. Chemical pathway proposed for 4-demethylation of 4-methylsterols.

CHAPTER II

METABOLISM OF LANOSTEROL BY A RECONSTITUTED CYTOCHROME P-450-CONTAINING SYSTEM[1]

SUMMARY

NADPH oxidation catalyzed by a reconstituted system consisting of $P\text{-}450_{14DM}$ and NADPH-cytochrome P-450 reductase both purified from semi-anaerobically grown cells of Saccharomyces cerevisiae was enhanced by lanosterol. Reduction of $P\text{-}450_{14DM}$ in the reconstituted system occurred at a significant rate only in the presence of lanosterol. In addition, lanosterol could induce a Type I spectral change in $P\text{-}450_{14DM}$. These results clearly indicated that lanosterol interacts with $P\text{-}450_{14DM}$ and increases the reactivity of the cytochrome with its reductase.

Incubation of lanosterol with the reconstituted system in the presence of NADPH and molecular oxygen resulted in the formation of a sterol metabolite. Coversion of lanosterol to the metabolite was dependent on both $P\text{-}450_{14DM}$ and NADPH-cytochrome P-450 reductase and rate of the metabolism was about 9.0 nmol lanosterol metabolized per min per nmol $P\text{-}450_{14DM}$. This metabolism of lanosterol was inhibited by metyrapone, SKF-525A and carbon monoxide.

Based on gas-chromatographic behaviours and mass spectrum, the metabolite was identified as 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol. It can, therefore, be concluded that the reconstituted system catalyzes 14α-demethylation of lanosterol in the presence of NADPH and molecular oxygen.

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[1] This work has been published by Yuri Aoyama and Yuzo Yoshida in Biochem. Biophys. Res. Commun., 82, 33-38 (1978) and ibid., 85, 28-34 (1978).

INTRODUCTION

As described in Chapter I, P-450$_{14DM}$ and NADPH-cytochrome P-450 reductase of Saccharomyces cerevisiae microsomes have been purified by Yoshida et al. (16) and by Aoyama et al. (13,17), respectively. In recent years, catalytic properties of indivisual molecular species of mammalian cytochrome P-450 have been studied with reconstituted systems consisting of purified preparations of cytochrome P-450 and the reductase (45,46). So, the author studied catalytic properties of P-450$_{14DM}$ with a reconstituted system consisting of purified preparations of the cytochrome (16) and the reductase (13,17). Since contri= bution of cytochrome P-450 to lanosterol demethylation by yeast microsomes has been suggested (28,31,41), metabolism of lanosterol by the reconstituted system was extensively studied, and it was found that the reconstituted system catalyzed 14α-demethylation of lanosterol in the presence of NADPH and molecular oxygen.

MATERIALS AND METHODS

Chemicals and Biochemicals: NADPH, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast Co., Tokyo. Glucose oxidase and catalase were the products of Boehringer Manheim GmbH. Emulgen 913 was a generous gift of Kao-Atras Co., Tokyo. Lanosterol was prepared by the preparative GLC with OV-17 column from a crude lanosterol preparation obtained from Nakarai Chemicals Co., Kyoto. DLPC was the product of Sigma Chemicals Co. 1% OV-17 on chromosorb W (80-100 mesh) was obtained from Wako Pure Chemicals Co., Osaka. Other chemicals were the purest reagents from commercial sources.

Cultivation of Yeast and Preparation of Microsomes: A wild-type strain of S. cerevisiae was precultivated semi-anaerbically at 30°C for 24 h in a

medium consisting of 1% glucose, 0.5% polypeptone and 1% yeast extract. The cells were harvested and suspended with distilled water to give an absorption at 650 nm of 1.14. The cell suspension was then inoculated to a growth medium consisting of 3% glucose, 0.5% polypeptone, 0.5% yeast extract and 0.5% KH_2PO_4 (7.5 ml of the innoculum per liter of the medium), and the cultivation was carried out semi-anaerobically at 30°C for 15 h. The calls were harvested, washed twice with cold distilled water, and suspended in 0.65 M mannitol. The thick suspension of the cells in 0.65 M mannitol was passed through a French pressure cell (Ohtake Works Co., Tokyo) at an out put pressure of 1200 to 1500 kg/cm^2. The cell-free suspension thus obtained was centrifuged at 10,000 x g for 20 min and the resulting supernatant was further centrifuged at 125,000 x g for 90 min. The precipitate from the second centrifugation was washed successively with 0.1 M potassium phosphate buffer, pH 7.2, con= taining 10 mM EDTA, and 0.1 M potassium phosphate buffer, pH 7.2. The washed precipitate was suspended in 0.1 M potassium phosphate buffer, pH 7.2, and used as the microsomal fraction.

Purification of $P\text{-}450_{14DM}$ and NADPH-Cytochrome P-450 Reductase from Semi-Anaerobically Grown Yeast Cells: $P\text{-}450_{14DM}$ was solubilized from microsomes of semi-anaerobically grown yeast cells with sodium cholate. The solubilized cytochrome was purified by ammonium sulfate fractionation and successive column chromatographies on AH-Sepharose 4B, Bio-Gel HT, and CM-Sephadex C-50 in the presence of Emulgen 913 (16). NADPH-cytochrome P-450 reductase of the same source was also solubilized with sodium cholate. The enzyme was then purified with ammonium sulfate fractionation followed by chromatographies on Hypatite C and DE-52 (13,17). The purified preparations were stored at -70°C until use.

Reconstitution of a $P\text{-}450_{14DM}$-Containing Electron-Transport System: A $P\text{-}450_{14DM}$-containing electron-tranport system was reconstituted by mixing

0.15 to 0.3 μM $P\text{-}450_{14DM}$ and 1 to 2 units of NADPH-cytochrome P-450 reductase in 2.0 ml of 0.1 M potassium phosphate buffer, pH 7.2. This reconstituted system usually contained trace (less than 0.001%) Emulgen 913 and 0.025% sodium cholate that came from the cytochrome and the reductase preparations, respectively.

Reduction of $P\text{-}450_{14DM}$ in the Reconstituted System: The reaction mixture consisting of the reconstituted system described above, 7.5 mM glucose, 2 units glucose oxidase, and 2600 catalase units catalase was placed in a spectrophotometer cuvette and bubbled with carbon monoxide. The mixture was preincubated at 30°C for 5 min to achieve anaerobiosis, and then the reaction was started by the addition of 0.15 mM NADPH by a mixing plunger apparatus. The increment of absorbance difference between 448 and 500 nm due to the formation of the reduced CO-complex of the cytochrome was followed in a Hitachi 156 dual-wavelength recording spectrophotometer.

Lanosterol Metabolism by the Reconstituted System: The reaction mixture for the assay of lanosterol metabolism by the reconstituted system consisting of the reconstituted system, 10 mM glucose 6-phosphate, 0.2 unit glucose-6-phosphate dehydrogenase and 13 nmol of lanosterol in micelles made from 80 nmol of DLPC. The reaction was run at 30°C under constant shaking. The reaction was stopped by the addition of 5 ml of 10% (w/v) KOH in methanol and the mixture was heated at 80°C for 60 min. Sterols in the saponified mixture were extracted with petroleum ether/diethyl ether (90/10, v/v). The solvent was evaporated and the residue was analyzed in a Shimadzu GC-mini 2 gas chromatograph equipped with a hydrogen-flame ionization detector. Sterols were separated through a glass column (2.6 mm x 1.0 m) packed with 1.0% OV-17 on chromosorb W (80-100 mesh) at 265°C using nitrogen as the carrier gas. Lanosterol 14α-demethylation activity was usually expressed by the conversion ratio of lanosterol to 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol calculated from the

gas chromatogram.

Analysis of Lanosterol Metabolite with GC-MS: Sterols were extracted from the reaction mixture as described above and subjected to the analysis with a Hitachi RMU-6MG integrated gas chromatograph-mass spectrometer. Sterols were separated at 240°C through a glass column (3 mm x 1 m) of 1% OV-17 (80-100 mesh). Helium was used as the carrier gas at an input pressure of 1.3 kg/cm^2. Scanning rate and ionizing energy of the mass spectrometer were set at 100 mass/sec and 20 eV, respectively.

Other Analytical Methods: Protein was determined by the method of Lowry et al. (47) using bovine serum albumin as standard. Optical absorption spectrum was measured at room temperature in a Shimadzu UV-300 recording spectrophotometer equipped with a Shimadzu SAPCOM-1 spectral-data processor.

RESULTS AND DISCUSSION

Evidence for the Interaction of Lanosterol with $P\text{-}450_{14DM}$: A reconstituted system consisting of $P\text{-}450_{14DM}$ and NADPH-cytochrome P-450 reductase both purified from semi-anaerobically grown cells of S. cerevisiae catalyzed NADPH oxidation by molecular oxygen. The maximum rate of the NADPH oxidation was observed when lanosterol was added to the reaction mixture (Table II-I). In contrast, the rates of NADPH oxidation observed in the presence of some drugs which are known as substrates for hepatic microsomal cytochrome P-450 did not exceed that observed in the presence of DLPC alone. Although no NADPH oxidation was observed in the absence of the reductase, significant NADPH oxidation was observed without $P\text{-}450_{14DM}$. The NADPH oxidation observed in the absence of $P\text{-}450_{14DM}$ must be due to the autoxidation of the reductase, and this rate was comparable to that observed in the absence of lanosterol.

In agreement with the results described above, rapid reduction of $P\text{-}450_{14DM}$ by NADPH in the reconstituted system was observed only in the presence of

lanosterol (Fig. II-1, curve B). The apparent first-order rate constant of the cytochrome reduction calculated from curve B of Fig. II-1 was 21.8 min^{-1}. When only DLPC, the dispersing agent, was added to the reaction mixture the reduction proceeded with an apparent first-order rate constant of less than 2 min^{-1} (Fig. II-1, curve A). When lanosterol was displaced by other sterols such as cholesterol and ergosterol, no acceleration of the cytochrome reduction was observed (data not shown). These observations clearly indicated that the significant enzymatic redox turnover of $P\text{-}450_{14DM}$ in the reconstituted system occurs only in the presence of lanosterol. It is, therefore, highly likely that lanosterol binds to $P\text{-}450_{14DM}$ as its substrate and increases the reactivity of the cytochrome with its reductase as in the case of the binding of camphor to cytochrome $P\text{-}450_{cam}$ (48).

Lanosterol induced a Type I spectral change in $P\text{-}450_{14DM}$, and the magnitude of this spectral change depended on lanosterol concentration (Fig II-2). This result provides direct evidence for the binding of lanosterol to the cytochrome and supports the above-mentioned possibility that lanosterol is a substrate for $P\text{-}450_{14DM}$. However, the extent of spin-state change was considerably small and most of $P\text{-}450_{14DM}$ was still in the low-spin state even in the presence of a saturating concentration of lanosterol (Spectrum 8 of Fig. II-2). As described above, the enzymatic reduction rate of $P\text{-}450_{14DM}$ was markedly increased by the addition of lanosterol and more than 60% of $P\text{-}450_{14DM}$ underwent rapid reduction in the presence of 13.5 μM lanosterol. However, the spectrophotometrically assumed high-spin content at this lanosterol concentration was less than 10% (Fig. II-2). These facts seem to indicate that the partial spin-state change of $P\text{-}450_{14DM}$ is not due to the binding of lanosterol with a small portion of the cytochrome but results from the occurrence of the apparent spin-state equilibrium of the lanosterol-bound form. Such an incomplete spin-state change upon binding with a substrate has been

reported for some microsomal cytochrome P-450 from mammals (49,50).

Metabolism of Lanosterol by the Reconstituted System: Lanosterol dispersed by DLPC was incubated with the reconstituted system in the presence of NADPH and molecular oxygen, as described in MATERIALS AND METHODS. Sterols extracted from the reaction mixture were analyzed with GLC through a column of 1% OV-17. Sterols extracted from the reaction mixture after 15 min incubation showed two peaks in the chromatogram (Curve A of Fig. II-3). The first peak (peak I) was identified as lanosterol, the substrate, by comparing its retention time and mass spectrum with those of authentic lanosterol. The second one (peak II) which showed a relative retention time to lanosterol of 1.10 was not observed in the chromatogram of sterols from the unincubated reaction mixture (Curve B of Fig II-3). The relative intensity of peak II to peak I was increased depending on the reaction time (data not shown). Moreover, peak II did not appear when any one of $P\text{-}450_{14DM}$, the reductase or NADPH was omitted from the reaction mixture (data not shown). Based on these facts, it can be suggested that peak II represents the metabolite formed from lanosterol by the reconstituted system.

As described in the following section, the metabolite was identified as 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol, a C-29 sterol. So, the ratio of the area of peak II to that of peak I in a gas chromatogram is considered to be almost equivalent to the molar ratio of the metabolite to lanosterol. Accordingly, the rate of metabolism of lanosterol by the reconstituted system can be calculated from the initial amount of lanosterol and the conversion ratio of lanosterol to the metabolite as described in MATERIALS AND METHODS. As shown in Fig. II-4, the rate of the lanosterol metabolism increased when the amount of $P\text{-}450_{14DM}$ was increased in the reconstituted system in the presence of enough cytochrome P-450 reductase, and an apparent turnover of the metabolism was calculated to be 9.0 nmol metabolite formed per min per

nmol $P\text{-}450_{14DM}$.

The metabolism of lanosterol was inhibited by metyrapone, SKF-525A and carbon monoxide indicating that this is a typical cytochrome P-450-dependent reaction.

Identification of the Metabolite and a Possible Mechanism of the Lanosterol Metabolism: Figure II-5A shows a mass spectrum of the metabolite (peak II of Curve A of Fig. II-3). The metabolite showed the molecular ion (M^+) at m/z 410 together with two characteristic fragment ions at m/z 395 and 377. The m/z values of these additional peaks indicated that they were assignable to M^+-CH_3 (m/z 395) and $M^+-CH_3-H_2O$ (m/z 377) both characteristic for sterols (51). It can thus be concluded that the metabolite is a sterol having a molecular weight of 410. As shown in Fig. II-5B, the mass spectrum of lanosterol showed three distinct peaks at m/z 426 (M^+), 411 (M^+-CH_3) and 393 ($M^+-CH_3-H_2O$). So, it is clear that one carbon and four hydrogen atoms were removed from lanosterol upon its conversion to the metabolite. In the mass spectrum of lanosterol (Fig. II-5B), M^+-CH_3 was the most intense peak, but in that of the metabolite, the most abundant peak was the M^+ as shown in Fig. II-5A. The intence M^+-CH_3 peak of the mass spectrum of lanosterol should be due to the presence of the two equivalent readily dissociable methyl groups (C-19 and C-32) at the arylic position of $\Delta^{8(9)}$. So, the metabolite seemed to have lost either C-19 or C-32 of lanosterol. It is now known that retention time of sterols in GLC is generally increased as a methyl group is added to the molecule (52). However, it is reported (52) that the introduction of 14α-methyl group into $\Delta^{8(9)}$-sterol exceptionally decreases its retention time in GLC. As shown in Fig. II-3, the retention time of the metabolite was larger than that of lanosterol. So, the metabolite does not contain the 14α-methyl group. Accordingly, it is concluded that lanosterol lost the 14α-methyl group (C-32) during the metabolism by the reconstituted system and at the same time one double bond was introduced into the molecule. The enzyme preparations used in the reconsti=

tuted system were highly purified (13,16,17). So, it is difficult to consider that the demethylation and the dehydrogenation occurred independently from each other. Moreover, in recent years, the chemical mechanism of lanosterol 14α-demethylation has been studied and it has been revealed that the 14α-methyl group is oxidatively removed as formic acid and at the same time a double bond is introduced between C-14 and C-15 (see Scheme I-3) (26,32,33). It can thus be concluded that the metabolite of lanosterol is 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol which has been assigned for the product of lanosterol 14α-demethylation (26,32,33,53,54). This in turn suggests that the reconstituted system can catalyze the entire process of lanosterol 14α-demethylation consisting of three oxygenations shown in Scheme I-3. If this is true, three moles of NADPH should be consumed as one mole of lanosterol is demethylated. Unfortunately, however, the stoichiometry between lanosterol demethylation and NADPH oxidation could not be determined because considerable amount of hydrogen peroxide was produced by uncoupling of P-450$_{14DM}$ and the amount of hydrogen peroxide could not be determined exactly due to its unexpected degradation in the reaction mixture for unknown reasons.

Recently, it was reported by Gibbons et al. (55) on lanosterol 14α-demethylation by hepatic microsomes that cytochrome P-450 catalyzed only C-32 hydroxylation and further metabolism of the 32-hydroxylated sterol was mediated by another CO-insensitive enzyme(s). This report is seemingly inconsistent with the above conclusion. However, the lanosterol 14α-demethylase system of hepatic microsomes has not yet been isolated and their conclusion was obtained only from the inhibitory effect of carbon monoxide on the metabolism of expected intermediates of the demethylation. So, more intensive study is required for the role of cytochrome P-450 in hepatic microsomal lanosterol 14α-demethylase system. In any way, the purified preparations of P-450$_{14DM}$ (16) and P-450 reductase (13,17) used in the present study were highly purified and therefore

it is evident that the cytochrome can catalyze the entire process of the 14α-demethylation.

Conclusion: The line of evidence described here undoubtedly indicates that P-450$_{14DM}$ of semi-anaerobically grown cells of S. cerevisiae catalyzes 14α-demethylation of lanosterol. The product of the demethylation is 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol, suggesting that the cytochrome may catalyze the three oxygenations shown in Scheme I-3.

TABLE II-I

OXIDATION OF NADPH WITH RECONSTITUTED SYSTEM CONSISTING OF P-450$_{14DM}$ AND NADPH-CYTOCHROME P-450 REDUCTASE

Reconstituted system consisting of 0.31 nmol P-450$_{14DM}$, 1.36 unit NADPH-cytochrome P-450 reductase and 0.15 mM NADPH. NADPH oxidation was run at 30°C and followed spectrophotometrically at 340 nm.

Reaction system	NADPH oxidation (nmol/min)
Reconstituted system [A]	0.89
[A] - reductase	0
[A] - P-450$_{14DM}$	1.11
Reconstituted system + DLPC (40 μM) [B]	1.11
[B] + lanosterol (12 μM)	4.89
[B] + aniline (100 mM)	0.89
[B] + benzphetamine (50 mM)	0.99
[B] + aminopyrine (80 mM)	1.00

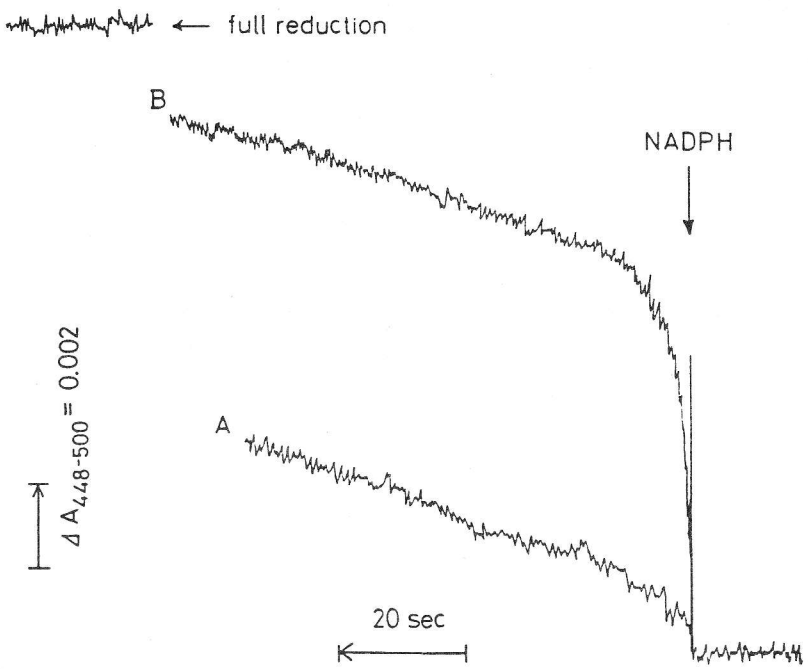


Fig. II-1. Effect of lanosterol on the reduction of P-450$_{14DM}$ in the recon= stituted system. Reduction of P-450$_{14DM}$ was measured in the presence (B) or absence (A) of lanosterol as described in MATERIALS AND METHODS. The amounts of P-450$_{14DM}$ and NADPH-cytochrome P-450 reductase were 0.32 nmol and 1.34 unit, respectively. Lanosterol (29.3 nmol) dispersed with 80 nmol DLPC was added to the reaction mixure in experiment B and 80 nmol DLPC was added in experiment A.

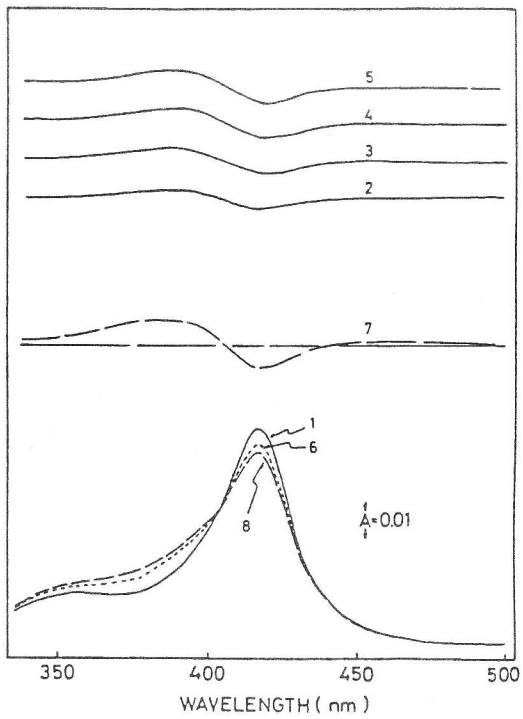


Fig. II-2. Lanosterol-induced spectral change in P-450$_{14DM}$. P-450$_{14DM}$ was disssolved in 100 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol and 0.04% Emulgen 913 at a concentration of 0.55 μM. After recording the absorption spectrum of the substrate-free from (Spectrum 1), P-450$_{14DM}$ was titrated with lanosterol dispersed with DLPC (lanosterol/DLPC = 1/3 in molar ratio) and the resulting difference spectra caliculated with the data processor were recorded (Spectra 2-5). The absolute absorption spectrum observed in the presence of the highest concentration of lanosterol used was recorded (Spectrum 6). Then, the reciprocals of the absorbance difference between 385 and 419 nm ($1/\Delta A$) was plotted against the reciprocals of the apparent lanosterol concentrations to determine the maximum absorbance difference of the difference spectrum (ΔA_{max}). By using this ΔA_{max} value, the difference spectrum which should be observed at the infinite concentration of lanosterol (Spectrum 7) was calculated and depicted with the data processor. The estimated absolute absorption spectrum of the lanosterol-saturated P-450$_{14DM}$ (Spectrum 8) was then depicted by adding Spectrum 7 to Spectrum 1. The apparent lanosterol concentrations used in the titration were 4.1, 8.2, 12.3 and 16.4 μM for Spectra 2, 3, 4 and 5, respectively.

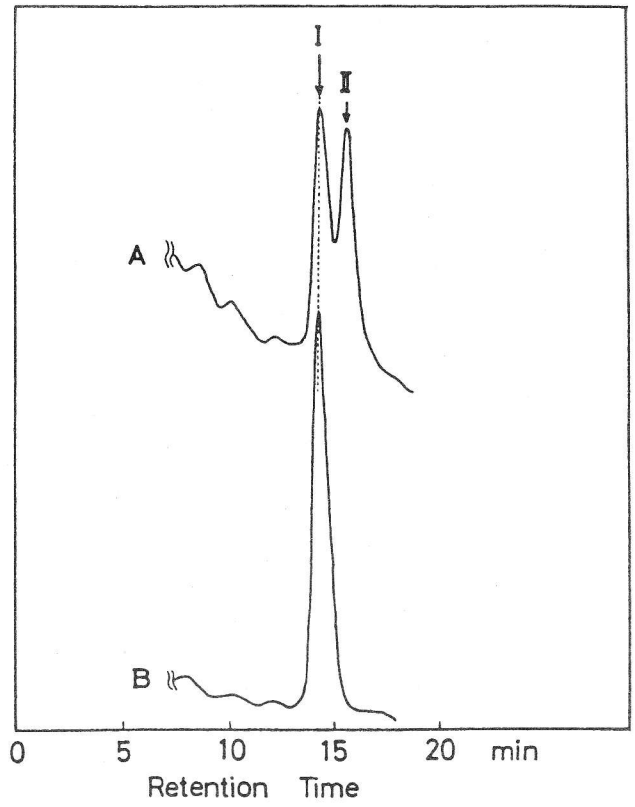


Fig. II-3. Gas chromatograms of sterols extracted from the reaction mixture. A; Lanosterol dispersed with DLPC was incubated at 30°C for 15 min in the reaction mixture described in MATERIALS AND METHODS. Sterols in the reaction mixture were analyzed with GLC as described in MATERIALS AND METHODS. B; Sterols extracted at time 0 from the reaction mixture were analyzed under the same conditions as A.

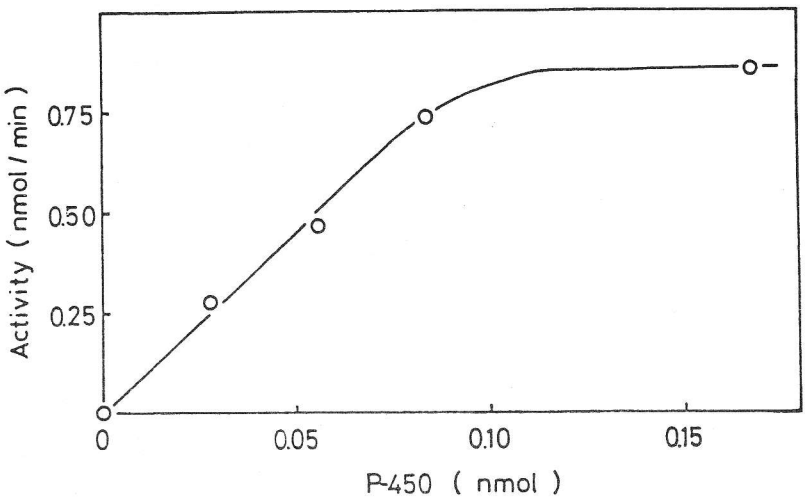


Fig. II-4. Dependency of the lanosterol metabolism on the amount of P-450$_{14DM}$ in the reconstituted system. The metabolism of lanosterol by the reconstituted system was measured as described in MATERIALS AND METHODS except that the amount of P-450$_{14DM}$ was varied as indicated.

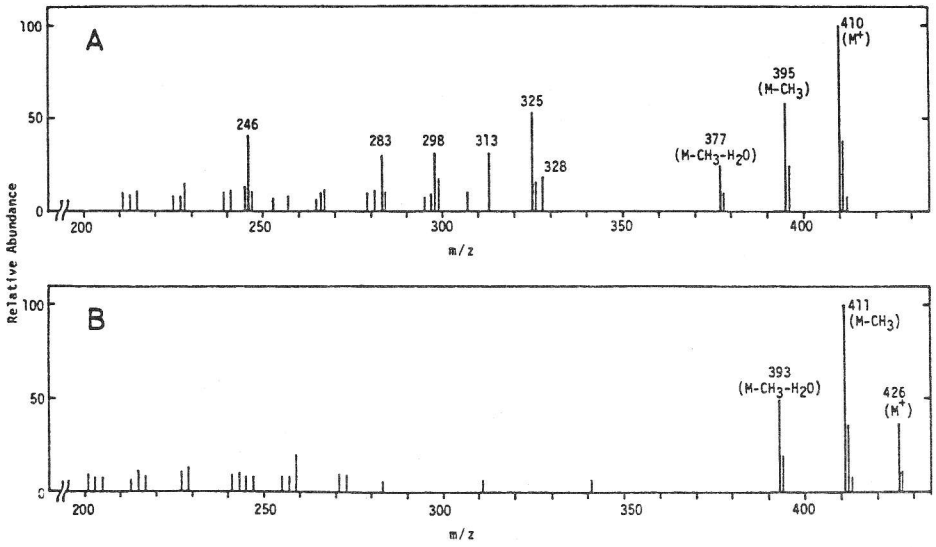


Fig. II-5. Mass spectra of the metabolite and lanosterol. A; The same sample as used in the experiment of Fig. II-3 was subjected to GC-MS as described in MATERIALS AND METHODS and the mass spectrum of peak II was recorded. B; Mass spectrum of lanosterol recorded under the same conditions as A.

CHAPTER III

EVIDENCE FOR THE INVOLVEMENT OF P-450$_{14DM}$ IN LANOSTEROL 14α-DEMETHYLATION OCCURRING IN MICROSOMES[2]

SUMMARY

Intact microsomes of semi-anaerobically grown Saccharomyces cerevisiae cells converted endogenous lanosterol to 4,4-dimethylzymosterol in the presence of NADPH, molecular oxygen and cyanide. Lanosterol 14α-demethylase activity was observed also in the microsomes from aerobically grown yeast cells which had been reported to contain little cytochrome P-450. This metabolism of lanosterol was inhibited by well-known cytochrome P-450 inhibitors such as carbon monoxide, metyrapone and SKF-525A. These observations indicate that cytochrome P-450 contributes to lanosterol 14α-demethylation occurring in microsomes regardless of growing conditions of yeast.

Antibodies to P-450$_{14DM}$ inhibited lanosterol 14α-demethylation by microsomes of semi-anaerobically and aerobically grown yeast. In Ouchterlony double-diffusion test, the antibodies formed a single precipitin line with the cholate-solubilized microsomes from both semi-anaerobically and aerobically grown yeast cells, and these precipitin lines fused with that formed by the purified P-450$_{14DM}$. It can thus be concluded that lanosterol 14α-demethlation in yeast is surely catalyzed by P-450$_{14DM}$.

Presence of cytochrome P-450 in microsomes of aerobically grown yeast was confirmed by the second-derivative spectrophotometry.

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[2]A part of this work has been published by Yuri Aoyama, Takako Okikawa and Yuzo Yoshida in Biochim. Biophys. Acta, 665, 596-601 (1981).

INTRODUCTION

As described in Chapter II, the reconstited system consisting of P-450$_{14DM}$ and NADPH-cytochrome P-450 reductase catalyzed 14α-demethylation of lanosterol. However, there is no confirmative evidence supporting that this demethylase system is physiologically functional in lanosterol 14α-demethylation occurring in yeast microsomes. Moreover, it has been reported that cytochrome P-450 present in semi-anaerobically grown cells of S. cerevisiae disappeared upon aerobic adaptation (6). If the cytochrome is essentially absent in aerobically grown yeast cells, lanosterol 14α-demethylation in these cells must occur in a different way from that in the semi-anaerobically grown cells, or lanosterol 14α-demethylation by the reconstituted system described in Chapter II should not be essential for ergosterol biosynthesis in yeast.

In this chapter, the author describes evidence indicating the involvement of P-450$_{14DM}$ in lanosterol 14α-demethylation occurring in both semi-anaerobically and aerobically grown cells of S. cerevisiae.

MATERIALS AND METHODS

Chemicals and Biochemicals: Freund's complete adjuvant was obtained from Nakarai Chemicals Co., Kyoto. Agarose A was the product of Pharmacia Fine Chemicals Co. Other chemicals and biochemicals were the same reagents as those described in the preceding chapter.

Cultivation of Yeast and Preparation of Microsomes: Method for cultivation of semi-anaerobically grown cells of S. cerevisiae was described in the preceding chapter. For aerobic growth, the yeast cells were precultivated at 30°C for 24 h under constant shaking in a medium consisting of 1% glucose, 0.5% polypeptone, 0.5% yeast extract and 0.5% KH_2PO_4. The inoculum thus obtained was seeded as described in the preceding chapter to a growth medium

of the same composition as above. Then, the cells were aerobically grown at 30°C for 16 h under constant shaking.

Microsomes of both semi-anaerobically and aerobically grown yeast cells were prepared as described in the preceding chapter.

Assay of Lanosterol 14α-Demethylase Activity in Microsomes: Microsomal fraction prepared as above usually contained considerable amounts of lanosterol. Since this endogenous lanosterol was demethylated by the microsomes more effec= tively than exogenously added one, the endogenous lanosterol was used as the substrate. The reaction mixture (2.0 ml) contained 0.1 M potassium phosphate buffer, pH 7.2, an NADPH generating system consisting of 0.15 mM NADPH, 10 mM glucose 6-phosphate and 0.2 unit glucose-6-phosphate dehydrogenase, 0.2 mM EDTA, 1 mM KCN and 6 to 10 mg protein of microsomes. Cyanide included in the mixture prevented further metabolism of 4,4-dimethylzymosterol, the product of the 14α-demethylation in microsomes (see Chapter V and Ref. 41). The re= action was carried out at 30°C under constant shaking and stopped by the addi= tion of 5 ml of 10% KOH in methanol. The mixture was heated at 80°C for 60 min, and sterols were extracted three times with 12.5 ml each of petroleum ether/diethyl ether (9/1). The extract was dehydrated with Na_2SO_4 and the solvent was evaporated off. The residue was dissolved in chloroform and subjected to TLC on silica gel plates using n-hexane/ethylacetate/acetic acid (5/5/1) as the developing solvent. This chromatography separated lanosterol and its 14-demethylated derivatives from other sterols but could not separate 4,4-dimethylzymosterol from lanosterol. The spot corresponding to lanosterol was scraped off and extracted with chroloform/methanol (2/1) and subjected to GLC as described in the preceding chapter. Lanosterol 14α-demethylase ac= tivity was calculated by essentially the same method as described in the pre= ceding chapter except that the metabolite was not 4,4-dimethyl-5α-cholesta-8,14, 24-trien-3β-ol but 4,4-dimethylzymosterol which showed a relative retention time

to lanosterol of 1.05 in the GLC.

Other Analytical Methods: Mass fragmentography of lanosterol metabolite was carried out in a JEOL LMS integrated GC-MS equipped with a multi-ion detector. Sterols were separated at 240°C through a glass column (3 mm x 1 m) packed with 2% OV-17 (80-100 mesh) and monitored at m/z 410 and 412. The second-derivative spectra of aerobically grown yeast microsomes were mesured in a Shimadzu UV-300 recording spectrophotometer equipped with a SAPCOM-1 spectral-data processor.

Immunochemical Techniques: Antibodies to P-450$_{14DM}$ were raised in a rabbit using a purified preparation of the cytochrome from semi-anaerobically grown cells of S. cerevisiae (16) as the antigen. The antigen (5.3 mg protein) was injected subcutaneously with Freund's complete adjuvant to the rabbit at four points on the back. Four and six weeks after the first injection, the rabbit was boosted with 2.1 and 1.0 mg protein, respectively, of the antigen. One week after the last injection, blood was collected from the carotid and serum was separated from the clotted blood. γ-Globulin fraction was prepared from the serum by ammonium sulfate fractination (56). The control γ-globulin fraction was prepared as above from an unimmunized rabbit.

Ouchterlony double-diffusion test was performed with an agarose plate consisting of 50 mM potassium phosphate buffer, pH 7.2, 20% glycerol, 0.2% Emulgen 913, 1% sodium cholate, 0.02% sodium azide and 1% agarose A. Immuno= diffusion was carried out at room temperature for 36 h and precipitin lines were stained with Coomassie blue after removal of unprecipitated proteins.

RESULTS AND DISCUSSION

Metabolism of Lanosterol in Microsomes from Semi-Anaerobically and Aerobically Grown Yeast: Microsomes prepared from semi-anaerobically grown cells of S. cerevisiae contained 10 to 20 nmol of lanosterol per mg of protein.

Upon aerobic incubation of the microsomes at 30°C in the presence of a NADPH generating system, the endogenous lanosterol was converted to some demethylated products. When cyanide, a potent inhibitor for methyl sterol 4-demethylase (see Chapter V and Ref. 41), was added to the reaction mixture, the endogenous lanosterol was converted to only one metabolite which showed a relative retention time to lanosterol of 1.05 in the GLC (Fig. III-1). This metabolite was identified as 4,4-dimethylzymosterol from its GLC behaviour (52,57,58) and mass spectrum (TABLE III-I).

Formation of 4,4-dimethylzymosterol from the endogenous lanosterol in microsomes was inhibited by carbon monoxide, metyrapone and SKF-525A which are known to be potent inhibitors for cytochrome P-450 (TABLE III-II). This metabolism of the endogenous lanosterol was also inhibited by menadione (TABLE III-II) which is known to form an electron shunt from NADPH-cytochrome P-450 reductase to molecular oxygen (17). These facts suggested that the metabolism of the endogenous lanosterol to 4,4-dimethylzymosterol was catalyzed by an enzyme system containing of cytochrome P-450 and its reductase. As described in the preceding chapter, the metabolite of lanosterol formed by the reconstituted system consisting of $P\text{-}450_{14DM}$ and NADPH-cytochrome P-450 reductase was not 4,4-dimethylzymosterol but 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol (M_r 410) which showed a relative retention time to lanosterol of 1.10 in the GLC (see Fig. II-3). Formation of a trace amount of this dimethylcholestatrienol in intact microsomes could be detected by mass fragmentography of lanosterol metabolites (Fig. III-2). This observation indicated that the dimethylcholestatrienol was formed also in intact microsomes, and suggested the possibility that microsomes contained a certain enzyme reducing the dimethylcholestatrienol to 4,4-dimethylzymosterol. Presence of such a reductase was suggested for the hepatic microsomal lanosterol demethylase system (30). In addition, a sterol Δ^{14}-reductase reacting on ergosta-8,14-dien-3β-ol was found in S. cerevisiae

microsomes (59).

Microsomes prepared from aerobically grown cells of S. cerevisiae contained 2 to 3 nmol of lanosterol per mg of protein. Upon incubation at 30°C in the presence of NADPH, molecular oxygen and cyanide, the endogenous lanosterol was effectively converted to 4,4-dimethylzymosterol (data not shown). As shown in Table III-III, this conversion did not occur when either NADPH or molecular oxygen was omitted from the reaction system. Moreover, this conversion was inhibited by carbon monoxide (TABLE III-III). These observations suggested the occurrence of the cytochrome P-450-dependent lanosterol 14α-demethylation in microsomes from aerobically grown yeast. This in turn leads to the conclusions that lanosterol 14α-demethylation in yeast is catalyzed generally by cytochrome P-450 regardless of its growth conditions and that cytochrome P-450 must be present in microsomes of aerobically grown yeast though there is no spectrophotometric evidence for the presence of the cytochrome in aerobically grown yeast.

Rate of lanosterol demethylation observed in microsomes of semi-anaerobically and aerobically grown yeasts were calculated to be 1.02 nmol/min/mg protein and 0.017 nmol/min/mg protein, respectively. This fact indicated that the content of lanosterol 14α-demethylase in aerobically grown yeast microsomes is very low as compared with that in semi-anaerobically grown ones. However, this activity of the microsomes from aerobically grown yeast was still higher than the over all rate of conversion of lanosterol to zymosterol (0.004 to 0.006 nmol/min/mg protein) by the aerobically-adapted cells of semi-anaerobically grown yeast having a high sterol biosynthetic activity (41).

Immunochemical Evidence for the Involvement of $P\text{-}450_{14DM}$ in Lanosterol 14α-Demethylation in Yeast Microsomes: Antibodies to $P\text{-}450_{14DM}$ were raised in a rabbit. Figure III-3 shows the result of Ouchterlony double-diffusion test of the antibodies by using cholate-solubilized microsomes from aerobically

and semi-anaerobically grown yeast and purified P-450$_{14DM}$ as the antigens. The antibodies formed a single precipitin line with the purified P-450$_{14DM}$ (wells A, D and E), the cholate-solubilized microsomes of semi-anaerobically grown yeast (well B) or the cholate-solubilized microsomes of aerobically grown yeast (wells c and F). These precipitin lines fused with one another indicating that the antibodies reacted specifically with P-450$_{14DM}$. In addition, it is noteworthy that the result shown in Fig. III-3 provides immunochemical evidence for the presence of P-450$_{14DM}$ in microsomes of aerobically grown yeast. The precipitin line formed with the cholate-solubilized microsomes of aero= bically grown yeast (wells C and F) was weaker than that formed with the semi-anaerobically grown yeast microsomes (well B), though the amount of protein in wells C and F was larger than that in well B. This fact suggested that the P-450$_{14DM}$ content in the microsomes of aerobically grown yeast is lower than that in semi-anaerobically grown ones in agreement with the conclusion of the above section based on the lanosterol demethylase activity.

When microsomes from the semi-anaerobically grown yeast were preincubated with various amounts of the antibodies and then assayed for lanosterol 14α-demethylation, a clear dose-dependent inhibition was observed (Fig. III-4). The antibodies also inhibited lanosterol 14α-demethylation by aerobically grown yeast microsomes (Fig. III-5). Preincubation of the microsomes with the control γ-globulin resulted in no inhibition (Fig. III-4 and III-5). These results indicated that lanosterol 14α-demethylation occurring in yeast micro= somes was catalyzed by P-450$_{14DM}$ not only in semi-anaerobically grown cells but also in aerobically grown ones. It can, therefore, be concluded that P-450$_{14DM}$ is surely functional in intact microsomes as lanosterol 14α-demethylase.

Spectrophotometric Evidence for the Presence of Cytochrome P-450 in Microsomes of Aerobically Grown Yeast: Presence of P-450$_{14DM}$ in microsmes of aerobically grown yeast was confirmed both enzymatically and immunochemically.

However, it has been reported (5,6,60,61) that no spectrophotometric evidence was obtained for the presence of cytochrome P-450 in aerobically grown yeast cells. Actually, an absorption band around 450 nm characterisric for cytochrome P-450 was not observed in reduced-CO difference spectrum of microsomes from aerobically grown yeast cells (Fig. III-6B) and the spectrum was characteristic of the reduced-CO difference spectrum of cytochrome oxidase (62). So, to obtain spectral evidence for the presence of cytochrome P-450 in the microsomes of aerobically grown yeast, a second-derivative absorption specrum of the microsomes was recorded by means of a Shimadzu SAPCOM-1 spectral-data processor. The second-derivative spectrum of the reduced preparation (solid line of Fig. III-6A) showed absorption bands corresponding to the cytochromes of the respiratory chain due to the contaminating small fragments of mitochondria. Upon addition of carbon monoxide to the reduced sample, the absorption band at 445 nm disappeared, the signal intensity at 430 nm incrased, and new absorption bands appeared at 443 (shoulder) and 450 nm (broken line of Fig. III-6A). The disappearance of the 445 nm band and the increment of the signal intensity at 430 nm indicated the conversion of the reduced cytochrome oxidase to its carbon monoxide complex, and the newly appeared shoulder at 443 nm may correspond to the shoulder observed in the absorption spectrum of the reduced-CO compound of purified ferric cytochrome a (62). The absorption band at 450 nm (indicated with the arrow in Fig. III-6A) which corresponds to the very faint shoulder at this wavelength in the absolute spectrum (broken line of Fig. III-6C) must be attributable to the chracteristic Soret band of the reduced-CO complex of cytochrome P-450. These observations provided the spectrophotometric evidence supporting the existence of cytochrome P-450 in the microsomal fraction of aerobically grown yeast. As described in the preceding sections, the content of cytochrome P-450 in these microsomes was lower than that in microsomes of semi-anaerobically grown yeast. Moreover,

the microsomal fraction of aerobically grown yeast was contaminated with significant amounts of the mitochondrial cytochromes (Fig. III-6). These should be the reasons why cytochrome P-450 could not be found in aerobically grown yeast microsomes by usual spectrophotometry.

Conclusion: Taken all observations together, it can be concluded that lanosterol 14α-demethylation occurring in yeast microsomes is actually catalyzed by a monooxgenase system containing $P\text{-}450_{14DM}$. This cytochrome exists in microsomes of yeast regardless of growth conditions of yeast though the content is varied significantly depending on these conditions.

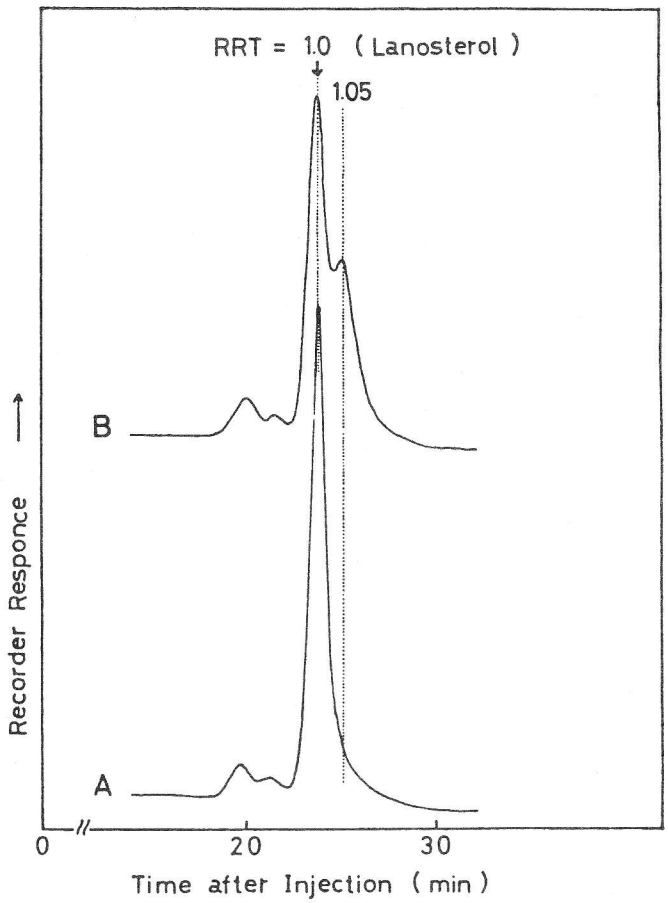


Fig. III-1. Gas-chromatographic detection of 14α-demethylation of the endoge= nous lanosterol in the microsomes from semi-anaerobically grown yeast. Lano= sterol 14α-demethylase activity was assayed as described in MATERIALS AND METHODS. Sterols extracted from the reaction mixture at time 0 (A) or after 20 min incubation (B) were subjected to GLC on a glass column packed with 1% OV-17.

TABLE III-I

GAS-CHROMATOGRAPH-MASS SPECTROMETRIC PARAMETERS OF LANOSTEROL, 4,4-DIMETHYL-5α-CHOLESTA-8,14,24-TRIEN-3β-OL AND THE METABOLITE

Compounds	Relative Retention Time	M^+ m/z (%)	M^+-CH_3 m/z (%)	$M^+-CH_3-H_2O$ m/z (%)
Lanosterol	1.00	426 (35)	411 (100)	398 (48)
4,4-Dimethyl-5α-cholesta-8,14,24-trien-3β-ol	1.10	410 (100)	395 (58)	377 (25)
The Metabolite	1.05	412 (100)	397 (41)	379 (19)

TABLE III-II

EFFECTS OF INHIBITORS ON LANOSTEROL 14α-DEMETHYLASE ACTIVITY IN MICROSOMES OF SEMI-ANAEROBICALLY GROWN YEAST

Lanosterol 14α-demethylase activity of microsomes was assayed as described in MATERIALS AND METHODS.

Inhibitor	Concentration	Inhibition (%)
CO	$CO:O_2$=90:10	51.1
	$CO:O_2$=95:5	100
Metyrapone	0.1 mM	57.3
SKF-525A	1.0 mM	100
Menadion	0.125 mM	62.1

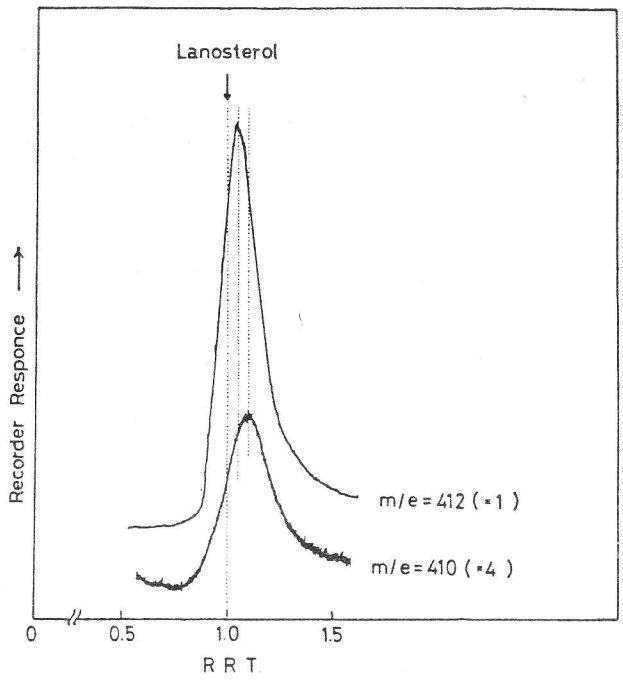


Fig. III-2. Mass-fragmentogram of lanosterol metabolites. The same sterol extract as used in Fig. III-1B was subjected to mass-fragmentography as described in MATERIALS AND METHODS.

TABLE III-III

COFACTOR REQUIREMENT AND CO-SENSITIVITY OF THE LANOSTEROL 14α-DEMETHYLASE ACTIVITY OF THE MICROSOMES FROM AEROBICALLY GROWN YEAST

Lanosterol 14α-demethylase activity was assayed as described in MATERIALS AND METHODS under the conditions shown in the Table.

Incubation mixture	Gas phase	Conversion (%)
Complete	Air	23.0
-NADPH	Air	0
Complete	N_2	2.1
Complete	$N_2:O_2$=95:5	17.4
Complete	$CO:O_2$= 95:5	0

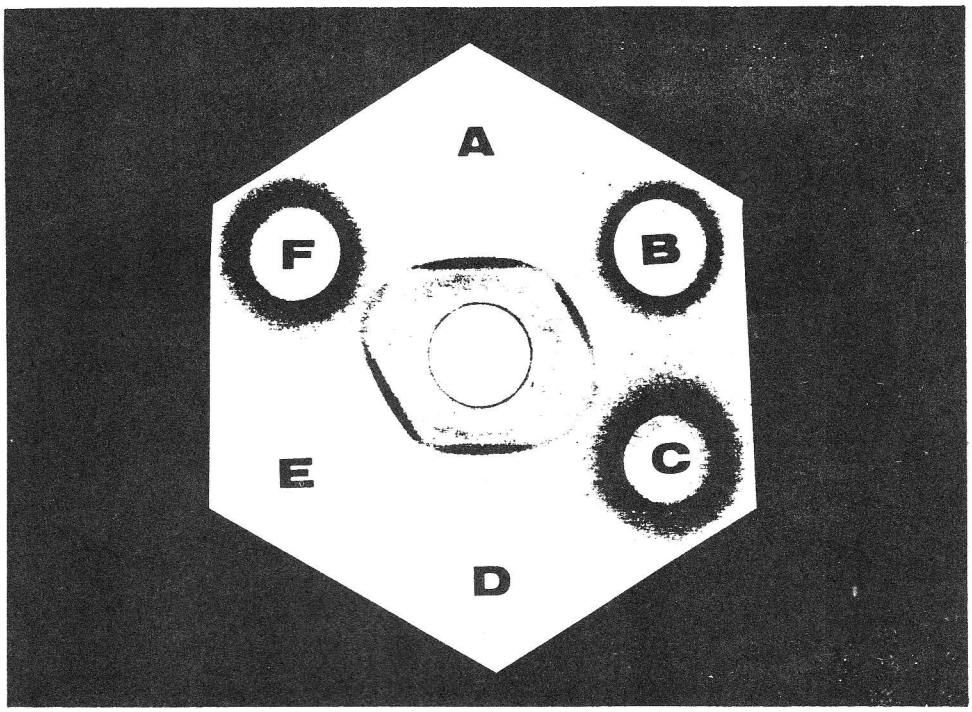


Fig. III-3. Immunochemical detection of cytochrome P-450 in the microsomal fraction of yeast by the Ouchterlony double-diffusion method. The center well contained 0.22 mg protein of the anti-cytochrome P-450 γ-globulin fraction. Wells A, D and E contained 0.18, 0.14 and 0.07 nmol, respsctively, of purified cytochrome P-450 used as the immunogen. Well B contained 1 mg protein of the cholate-solubilized microsomes of semi-anaerobically grown yeast. Both wells C and F contained 2 mg protein of the cholate-solubilized microsomal fraction of aerobically grown yeast. The conditions of immunodiffusion were described in MATERIALS AND METHODS.

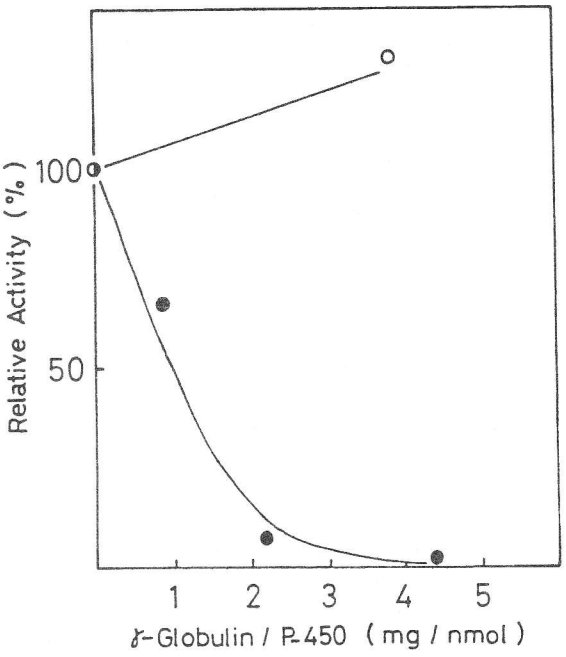


Fig. III-4. Inhibition of lanosterol 14α-demethylase activity of the microsomes from semi-anaerobically grown yeast cells by anti-cytochrome P-450 γ-globulin. The microsomes (0.66 nmol cytochrome P-450) were preincubated at 30°C for 15 min with various amounts of the anti-cytochrome P-450 γ-globulin (●) or the control γ-globulin (○). The lanosterol 14α-demethylase activity of the preincubated microsomes was assayed as described in MATERIALS AND METHODS.

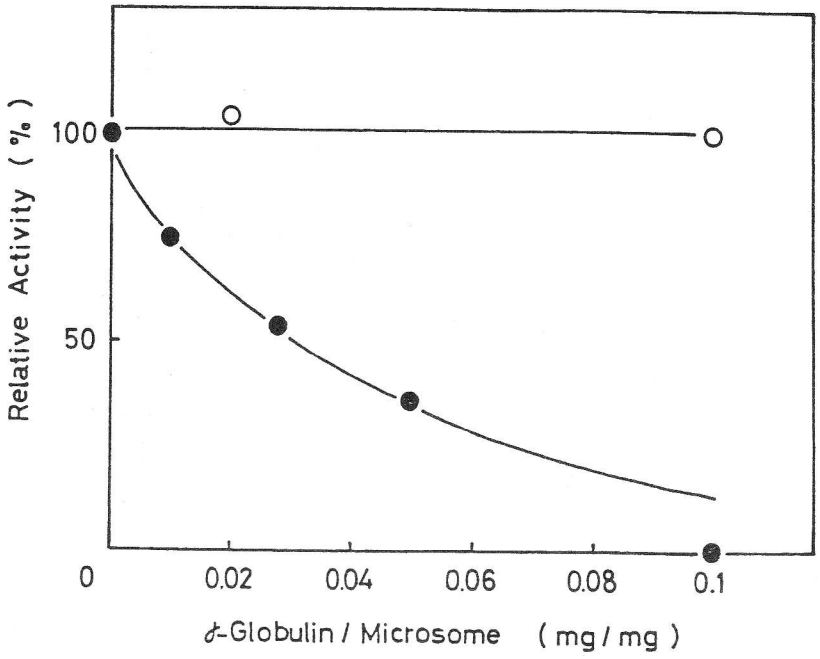


Fig. III-5. Inhibition of lanosterol 14α-demethylase activity of microsomes from aerobically grown yeast cells by anti-cytochrome P-450 γ-globulin. The microsomes (6.0 mg protein) were preincubated at 30°C for 15 min with various amounts of the anti-cytochrome P-450 γ-globulin (●) or the control γ-globulin (○). The lanosterol 14α-demethylase activity of the preincubated microsomes was assayed as described in MATERIALS AND METHODS.

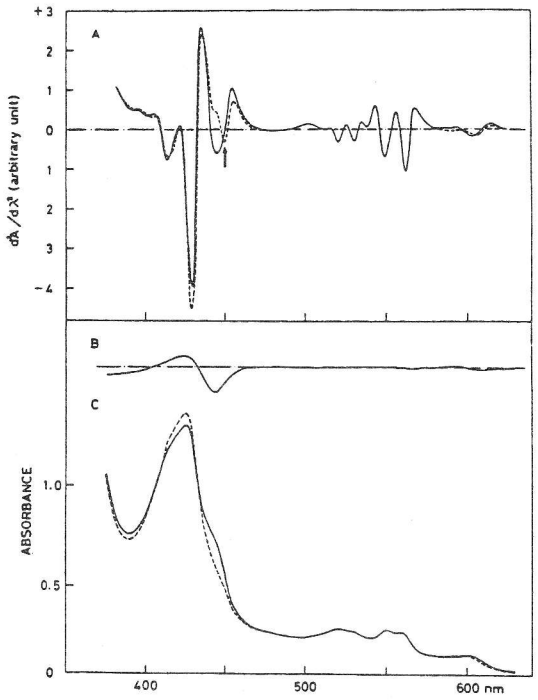


Fig. III-6. Detection of cytochrome P-450 in the microsomal fraction of aer= obically grown yeast by the second derivative spectrophotometry. The microsomes were suspended (10 mg protein/ml) in 0.1 M potassium phosphate buffer, pH 7.0, and apparent absolute spectra of the $Na_2S_2O_4$ reduced from (—) and the reduced-CO form (---) of the microsomes were recorded (C) using diluted milk as the reference and were stored in a digital memory of a Shimazu SAPCOM-1 spectral-data processor. Then, the spectra were converted to the second derivative spectra ($d^2A/d\lambda^2$ vs λ) (A) by means of the data processor. The sampling interval ($d\lambda$) was 0.6 nm and the spectra were scanned from right to left. B repesents an usual reduced-CO difference spectrum of the microsomes.

CHAPTER IV

BUTHIOBATE: A POTENT INHIBITOR FOR P-450$_{14DM}$[3]

SUMMARY

Buthiobate, a fungicide, which is known as an inhibitor of ergosterol biosynthesis in yeast, inhibited 14α-demethylation of lanosterol catalyzed by a reconstituted enzyme system consisting P-450$_{14DM}$ and NADPH-cytochrome P-450 reductase both purified from semi-anaerobically grown cells of Saccharomyces cerevisiae. Concentration of buthiobate necessary for 50% inhibition of the demethylation was 0.3 μM and this value was markedly lower than those of other inhibitors such as metyrapone and SKF-525A. Buthiobate induced a Type II spectral change in P-450$_{14DM}$ and inhibited lanosterol-dependent enzymatic reduction of the cytochrome. These facts indicated that buthiobate interacts with P-450$_{14DM}$ with high affinity and acts as potent inhibitor for the cyto= chrome.

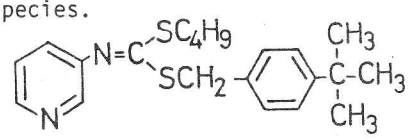
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[3]This work was orally presented by Yuri Aoyama, Yozo Yoshida, Shingo Hata, Tokuzo Nishino and Hirohiko Katsuki at 55th Annual Meeting of the Japanese Biochemical Society (October, 1982) in Osaka.

INTRODUCTION

It has been reported that buthiobate, a fungicide, inhibits the incorporation of radioactivity from [2-^{14}C]mevalonate into 14α-demethylated sterols by Saccharomyces cerevisiae cells and causes a marked accumulation of radioactive lanosterol (63). This fact strongly suggests that buthiobate inhibits 14α-demethylation of lanosterol in S. cerevisiae. As described in the preceding chapters, lanosterol 14α-demethylation in S. cerevisiae microsomes is catalyzed by cytochrome P-450.

In this chapter, the author examines the effects of buthiobate on P-450$_{14DM}$ and provides evidence indicating that this compound is a potent inhibitor for this cytochrome P-450 species.



BUTHIOBATE

MATERIALS AND METHODS

Buthiobate was kindly supplied by Dr. Toshiro Kato of Sumitomo Chemicals Co., Takarazuka. Buthiobate was added to the reaction mixtures as a dimethylsulfoxide solution and control experiments were carried out in the presence of a comparable amount of the solvent.

Other materials and methods used in the experiments of this chapter were essentially the same as those described in Chapter II.

RESULTS AND DISCUSSION

Inhibition by Buthiobate of Lanosterol 14α-Demethylase Activity: 14α-

Demethylation of lanosterol catalyzed by the reconstituted system described in Chapter II was inhibited by buthiobate as shown in Fig. IV-1. Buthiobate did not inhibit NADPH-cytochrome c reductase activity catalyzed by the cytochrome P-450 reductase used in the reconstituted system (data not shown). Accordingly, it was assumed that buthiobate interacted with P-450_{14DM} and inhibited lanosterol 14α-demethylation. The concentration of buthiobate required for 50% inhibition of the demethylation was calculated to be 0.3 μM from Fig. IV-1. This concentration was extremely low as compared with those of metyrapone (0.1 mM) and SKF-525A (0.2 mM), well known cytochrome P-450 inhibitors, necessary for 50% inhibition of the same activity.

Spectral Evidence for Binding of Buthiobate to P-450_{14DM}: Buthiobate caused a spectral change in S. cerevisiae cytochrome P-450 (Fig. IV-2A). This fact provided direct evidence for the binding of buthiobate to the cytochrome. The absorption spectrum of buthiobate-bound P-450_{14DM} was essentially superimposable on that of the pyridine complex of the cytochrome (data not shown) and also on those of the same complexes of mammalian cytochrome P-450 (49,64). So, it can be assumed that the pyridyl moiety of buthiobate interacted with the heme iron of the cytochrome. This spectral change was dependent on the amount of buthiobate and became maximal when one molecule of buthiobate was bound to one molecule of the cytochrome (Fig. IV-2B).

Upon reduction with $Na_2S_2O_4$, absorption spectrum of the buthiobate complex of P-450_{14DM} was changed as shown in Fig. IV-3. This fact indicated that buthiobate could bind also to the reduced from of the cytochrome. The buthiobate complex of the ferrous P-450_{14DM} was readily converted to the reduced-CO compound showing its Soret peak at 447 nm by brief bubbling of carbon monoxide (Fig. IV-3). This fact indicated that P-450_{14DM} was not denatured to P-420 by buthiobate.

Inhibition by Buthiobate of Lanosterol-dependent Enzymatic Reduction of

P-450$_{14DM}$: Buthiobate inhibited the lanosterol-dependent reduction of P-450$_{14DM}$ in the reconstituted system (Fig. IV-4), but the rate of reduction observed in the presence of excess buthiobate was not less than that observed in the absence of lanosterol (see Fig. II-1). In addition, buthiobate showed no effect on the chemical reduction of the cytochrome with $Na_2S_2O_4$. Therefore, the inhibition of P-450$_{14DM}$ reduction by buthiobate is likely to be due to the binding of the inhibitor to the cytochrome and resulting disapperance of the accelerating effect of lanosterol on the enzymatic reduction of the cytochrome. Buthiobate concentration necessary for 50% inhibition of the P-450$_{14DM}$ reduction was calculated to be 0.15 μM. This concentration was close to that required for 50% inhibition of lanosterol 14α-demethylation by P-450$_{14DM}$ (Fig. IV-1) indicating close relationship between inhibitory effects of buthiobate on the lanosterol demethylation and lanosterol-dependent enzymatic reduction of P-450$_{14DM}$.

Conclusion: Studies to elucidate detailed mechanism for the interaction of buthiobate with P-450$_{14DM}$, has not yet been completed. However, it is evident that buthiobate is a potent inhibitor for P-450$_{14DM}$.

Recently, Hata et al. (65) reported that Δ^{22}-desaturation of ergosta-5,7-dien-3β-ol in yeast microsomes, which has been considered to be a cytochrome P-450-dependent reaction (65,66), was not inhibited by buthiobate. This finding suggested the possibility that buthiobate inhibits specifically P-450$_{14DM}$. In addition, buthiobate caused Type II spectral change in P-450$_{14DM}$ at low concentrations (Curves C-F of Fig. IV-2A). So, buthiobate must be useful as a good indicator for P-450$_{14DM}$.

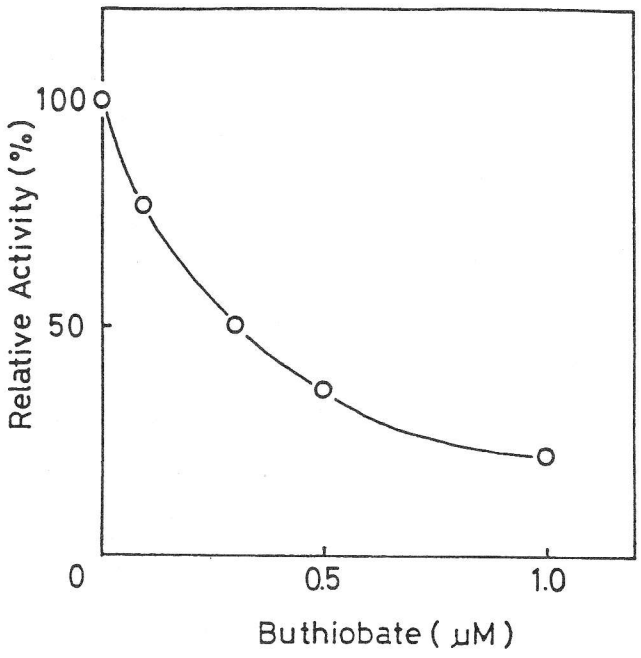


Fig. IV-1. Inhibition of lanosterol 14α-demethylase activity of the reconsti= tuted system by buthiobate. Lanosterol 14α-demethylase activity of the re= constituted system was assayed as described in Chapter II in the presence of indicated concentrations of buthiobate. Buthiobate was added as dimethyl= sulfoxide solution and dimethylsulfoxide concentration in the reaction mixture was fixed at 0.25% (v/v).

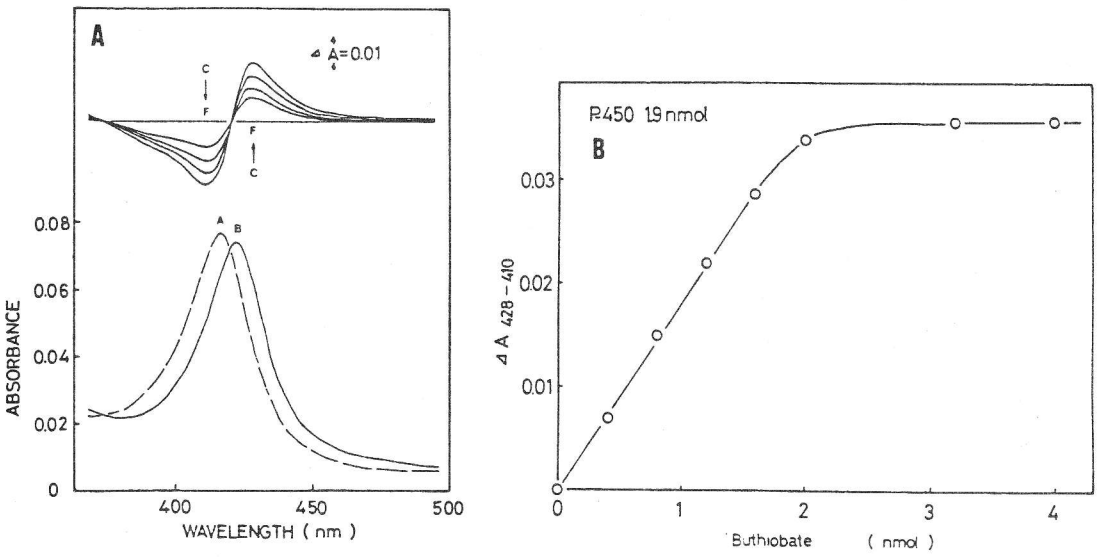


Fig. IV-2. Spectrophotometric titration of ferric P-450$_{14DM}$ with buthiobate.

A; P-450$_{14DM}$ (0.67 μM) in 0.1 M potassium phosphate buffer, pH 7.0, containing 20% glycerol was titrated with 0.29, 0.43, 0.57 and 1.07 μM of buthiobate and resulting spectral change of the cytochrome was recorded. Spectra C through F show the difference spectra observed during the titration. Lower half represents the absorption spectra of buthiobate-free (Spectrum A) and the buthiobate-bound (Spectrum B) forms of the cytochrome.

B; P-450$_{14DM}$ (1.9 nmol) was titrated with indicated amount of buthiobate and the intensity of the resalting difference spectra (see upper half of A) were plotted as the function of buthiobate concentration.

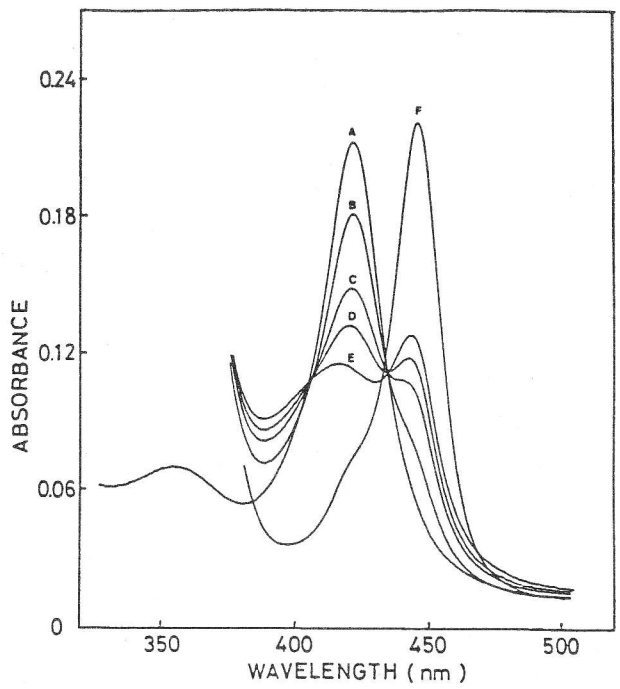


Fig. IV-3. Reduction of the buthiobate-P-450$_{14DM}$ complex with $Na_2S_2O_4$. P-450$_{14DM}$ (2.15 μM) in 0.1 M potassium phosphate buffer, pH 7.0, containing 20% glycerol was saturated with 8 μM buthiobate and reduced with $Na_2S_2O_4$. Spectrum A; before addition of $Na_2S_2O_4$. Spectra B-E; 2 (B), 4 (C), 6 (D) and 20 (E) min after the addition of $Na_2S_2O_4$. Spectrum F; after brief bubbling with carbon monoxide through the sample of Spectrum E.

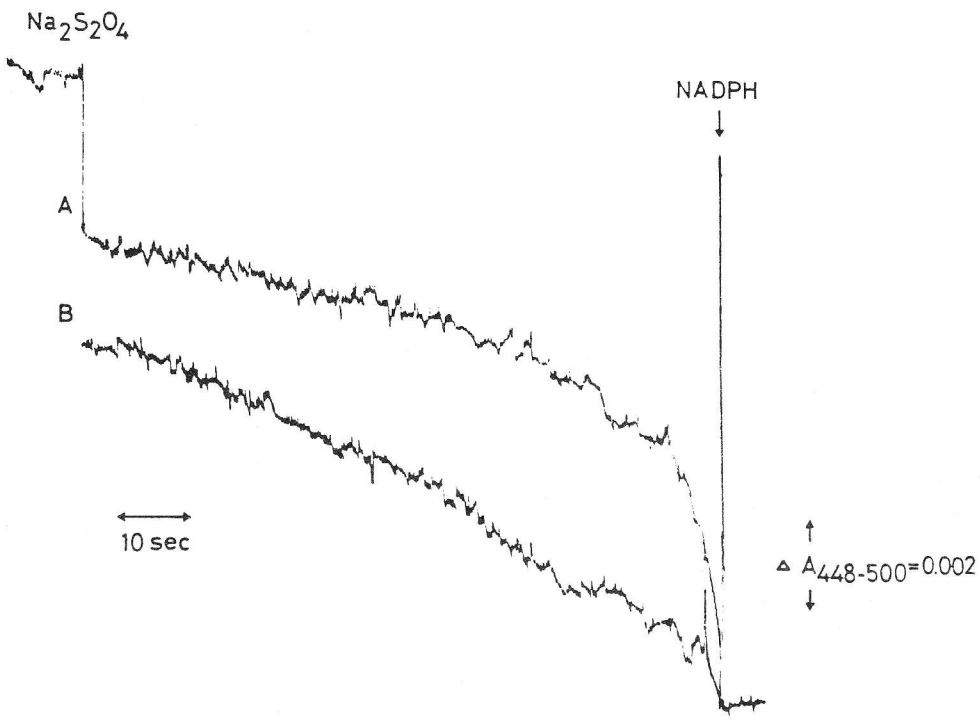


Fig. IV-4. Inhibition of lanosterol-dependent enzymatic reduction of $P\text{-}450_{14DM}$ in the reconstituted system by buthiobate. Reduction of $P\text{-}450_{14DM}$ was assayed as descrived in Chapter II in the presence (B) or absence (A) of buthiobate. Concentrations of $P\text{-}450_{14DM}$ and buthiobate were 0.16 μM and 1.0 μM, respectively. Buthiobate was dissolved in dimethylsulfoxide and dimethylsulfoxide concen= tration in the reaction mixture was fixed at 0.25% (v/v).

CHAPTER V

ELECTRON TRANSPORT SYSTEM CATALYZING 4-DEMETHYLATION OF 4,4-DIMETHYLZYMOSTEROL IN YEAST MICROSOMES[4]

SUMMARY

Liberation of $^{14}CO_2$ from one of the two methyl groups (C-30 and C-31) attached to the C-4 of [1,7,15,22,26,30-^{14}C]lanosterol in yeast microsomes was inhibited by cyanide but cyanide showed no effect on the lanosterol 14α-demethylase activity. The $^{14}CO_2$ liberation was also inhibited by antibodies to yeast cytochrome b_5 and by palmitoyl-CoA, a substrate for the cytochrome b_5-linked fatty acyl-CoA desaturase system of yeast microsomes. Neither the antibodies nor palmitoyl-CoA inhibited the conversion of lanosterol to 4,4-dimethylzymosterol. It can, therefore, be concluded that cytochrome b_5 and a cyanide-sensitive enzyme are involved in the 4-demethylation of 4,4-dimethylzymosterol, but not in the 14α-demethylation of lanosterol, by yeast microsomes. Accordingly, it can be assumed that the 4-demethylation is catalyzed by a cyanide-sensitive terminal enzyme, and cytochrome b_5 transfers reducing equivalents from NADPH to the terminal enzyme, as in the case of fatty acyl-CoA desaturation. Cyanide sensitivity of the 4-demethylation was, however, much higher than that of the desaturation.

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[4]This work has been published by Yuri Aoyama, Yuzo Yoshida, Ryo Sato, Markus Susani and Helmut Ruis in Biochim. Biophys. Acta, 663, 194-202 (1981).

INTRODUCTION

It is known that removal of the two methyl groups attached to C-4 of lanosterol occurs oxidatively in liver microsomes in the presence of NADPH, NAD^+ and molecular oxygen (Chapter I). Ohba et al. (41) reported that yeast microsomes also catalyzed oxidative removal of the three methyl groups attached to C-4 and C-14 of [1,7,15,22,26,30-^{14}C]lanosterol and one of them (C-30) was liberated as $^{14}CO_2$. In this process the methyl group at C-14 was first removed in a cyanide-insensitive reaction and then the two methyl groups at C-4 were removed by a cyanide-sensitive enzyme system (41). As described in Chapters II and III, the former is evidently catalyzed by $P\text{-}450_{14DM}$. However, the enzyme system responsible for the latter step has not yet been characterized. In this chapter, the author reports that the enzyme system consisting of cytochrome b_5 and a cyanide-sensitive enzyme participates in the 4-demethylation of 4,4-dimethylzymosterol, the product of lanosterol 14α-demethylation (Chapter III), by yeast microsomes.

MATERIALS AND METHODS

Chemicals and Biochemicals: N,N'-dibenzylethylenediamine D,L-[2-^{14}C] mevalonate (51 mCi/mmol) was obtained from Radiochemical Center, and palmitoyl-CoA (grade II) was from Sigma Chemicals Co. [1,7,15,22,26,30-^{14}C]lanosterol (hereafter referred simply to as [^{14}C]lanosterol) was prepared enzymatically from D,L-[2-^{14}C]mevalonate by the method of Gibbons and Mitropoulos (67) and purified by TLC on $AgNO_3$-silica gel plates using chloroform as solvent. The [^{14}C]lanosterol preparation thus purified was chemically and radiochemically pure when analyzed by GLC. Antibodies raised in rabbits against cytochrome b_5 of yeast (68) were supplied by Dr. H. Ruis of Viena University.

Cultivation of Yeast and Preparation of Microsomes: S. cerevisiae was

grown semi-anaerobically at 30°C in a medium containing 2% glucose, 0.5% polypeptone, 0.5% yeast extract and 0.5% KH_2PO_4. The cells, harvested at the late log phase, were suspended in 0.1 M potassium phosphate buffer, pH 6.2, containing 10% glucose (25 g wet cells/liter) and the suspension was shaken aerobically at 30°C for 90 min to increase sterol biosynthetic activity of the cells (41). The aerobically-adapted cells thus obtained were suspended (0.5 g wet cells/ml) in 0.1 M potassium phosphate buffer, pH 7.5, containing 0.05% glutathione and 0.35% nicotinamide and disrupted in a French pressure cell as described in Chapter II. The homogenate thus obtained was centrifuged at 10,000 x g for 20 min and the supernatant was again centrifuged at 125,000 x g for 90 min. Microsomes precipitated by the second centrifugation were washed once with the same buffer and suspended (30 mg protein/ml) in 0.1 M potassium phosphate buffer, pH 7.5, containing 3 mM glutathione. This preparation was stored at -80°C under nitrogen until use.

Enzyme Assays: The overall activity of lanosterol demethylation was assayed by determining the liberation of $^{14}CO_2$ from [^{14}C]lanosterol. The standard reaction mixture contained 80 mM potassium phosphate buffer, pH 7.5, yeast microsomes (about 4 mg protein), 30 nmol [^{14}C]lanosterol (15,000 cpm) dispersed with 80 nmol DLPC, 1.4 mM NAD^+, an NADPH generating system consisting of 0.3 mM NADPH, 10 mM glucose 6-phosphate and 0.15 unit glucose-6-phosphate dehydrogenase in a final volume of 1.0 ml. The tube containing reaction mixture was sealed with a rubber stopper to which a small cup containing 0.2 ml of Hyamine 10X was hung. The reaction was run aerobically at 30°C for 30 min with constant shaking. The reaction was stopped by injecting 0.5 ml of 0.1 N HCl through the rubber stopper, and mixture was shaken at 30°C for 30 min to ensure the absorption of evolved CO_2 to Hyamine 10X. The radioactivity of the Hyamine solution was counted with a liquid scintillation spectrometer. Antimycin A-insensitive NADH-cytochrome c reductase activity was assayed by the method of

Yoshida et al (9).

Radio-GLC of Lanosterol Metabolites: The reaction was carried out as above and stopped by adding 1.0 ml of 20% KOH in methanol to the reaction mixture, and the mixture was saponified at 80°C for 1 h. Sterols were extracted with petroleum ether, dried over anhydrous Na_2SO_4, and the solvent was evaporated. The radioactive sterols were analyzed by radio-GLC. The analysis was carried out in a Shimadzu GC-5A gas chromatogragh equipped with both a Shimadzu SG-22 radioisotope detector and a thermo-conductive detector. The glass column (4 mm x 1.5 m) was packed with 1.5% OV-17 on chromosorb W (80-100 mesh). Sterols were separated through this column at 240°C with nitrogen as carrier gas at a flow rate of 60 ml/min. Radioactive sterols thus separated were pyrrolyzed in a Shimadzu FNC-1A furnace and their radioactivity was detected with the radioisotope detector.

RESULTS AND DISCUSSION

Effect of Cyanide on $^{14}CO_2$ Liberation from [^{14}C]Lanosterol: The liberation of $^{14}CO_2$ from [^{14}C]lanosterol was inhibited by cyanide and the cyanide concentration giving 50% inhibition was estimated to be 0.08 mM (Fig. V-1). This result confirms the preliminary conclusion by Ohba et al. (41) that a cyanide sensitive enzyme participates in the methylsterol 4-demethylation by yeast microsomes and indicates its high sensitivity to cyanide. A marked sensitivity to cyanide has also been reported for the 4-demethylation of artifical sterols such as 4α-methyl-5α-cholesta-7-en-3β-ol by rat liver microsomes (36). In addition, the 4-demethylation of yeast (41) and rat liver system (35) exhibited the same cofactor requirement. These similarities between the hepatic and yeast sterol 4-demethylase systems seem to suggest that essentially the same mechanism is operating in the 4-demethylation of 4,4-dimethylzymosterol by yeast and mammals. Chemical mechanism of methyl sterol 4-demethylation occurring in

rat liver microsomes has extensively been studied by Gaylor and coworkers (see Chapter I). However, the mechanism by which reducing equivalents originated from NADPH are transferred to the terminal cyanide-sensitive enzyme is unknown not only for the yeast system but also for the mammalian one.

Effect of Anti-cytochrome b_5 Antibodies on $^{14}CO_2$ Liveration from [^{14}C] Lanosterol: Desaturation of long-chain fatty acyl-CoA's occurring in microsomes of yeast (12,68) or mammals (42,44) is known to be catalyzed by a cyanide-sensitive enzyme system (see Scheme I-1). In this system, cytochrome b_5 is established as an electron carrier supplying reducing equivalents from NAD(P)H to the cyanide-sensitive enzyme (desaturase) (12,42-44,68). Then, the possibility of involvement of cytochrome b_5 in the sterol 4-demethylation system of yeast microsomes was tested using antibodies raised in rabbits against yeast cytochrome b_5 (68) as a tool. The anti-cytochrome b_5 γ-globulin fraction used in this study formed a single precipitin line with both cholate-solubilized yeast microsomes and purified yeast cytochrome b_5 (18) in Ouchterlony double-diffusion and these two lines fused with each other (data not shown). As shown in Fig. V-2, the antibodies inhibited antimycin A-insensitive NADH-cytochrome c reductase activity, an activity which is catalyzed by the cooperative action of NADH-cytochrome b_5 reductase and cytochrome b_5 (9,14), of yeast microsomes. On the other hand, control γ-globulin fraction from an unimmunized rabbit did not affect the reductase activity (Fig. V-2). Based on these observations it is concluded that the antibodies inhibit the electron transfer reaction through cytochrome b_5.

As shown in Fig. V-3, the antibodies inhibited the formation of $^{14}CO_2$ from [^{14}C]lanosterol by yeast microsomes and the control γ-globulin fraction failed to inhibit the $^{14}CO_2$ formation. However, the inhibitory effect of the antibodies on the $^{14}CO_2$ formation was considerably less pronounced than that on the cytochrome c reductase activity (Fig. V-2), and no inhibition was observed

when the weight ratio of the γ-globulin to the microsomal protein was less than one. The reason for such a weak effect of the antibodies on the $^{14}CO_2$ is unclear. In any case, the specific inhibition by anti-yeast cytochrome b_5 antibodies provides evidence for a role of the cytochrome in the demethylation of lanosterol in yeast microsomes.

Inhibition by Palmitoyl-CoA of 4-Demethylation of 4,4-Dimethylzymosterol: Tamura et al. (12) have shown that addition of palmitoyl-CoA to NAD(P)H-reduced yeast microsomes under aerobic conditions caused a significant decrease in the steady-state reduction level of cytochrome b_5, due to an increased flow of reducing equivalents from the cytochrome to be utilized in the desaturation of the added palmitoyl-CoA. It is expected that this decrease in the reduction level of cytochrome b_5 should reduce the electron flow to be utilized for lanosterol demethylation, if the cytochrome is actually acting as an electron supplier for the terminal cyanide-sensitive enzyme responsible for the demethylation. As shown in Table V-I, palmitoyl-CoA actually inhibited the $^{14}CO_2$ formation from [^{14}C]lanosterol by yeast microsomes, confirming this expectation. This observation provides more support for the view that cytochrome b_5 is involved in lanosterol demethylation. Figure V-4 shows typical radio-gas chromatograms of sterol metabolites from [^{14}C]lanosterol in the presence and absence of palmitoyl-CoA. In the radio-gas chromatogram of sterols extracted from the reaction mixture after incubation at 30°C for 30 min in the absence of palmitoyl-CoA, four distinct radioactive peaks (termed peaks 1 through 4) were detected (Fig. V-4B). Peaks 1, 2, 3 and 4 could be identified as zymosterol, 4-methylzymosterol, lanosterol and 4,4-dimethylzymosterol, respectively, from their retention times (41,57,58). As expected, the radio-gas chromatogram of unsaponifiable materials extracted at zero time of the incubation exhibited only peak 3, i.e. lanosterol (Fig. V-4C). When incubation was carried out in the presence of 0.1 mM palmitoyl-CoA which caused about 70% inhibition of the $^{14}CO_2$

liberation from [^{14}C]lanosterol, the formation of zymosterol (peak 1) and 4-methylzymosterol (peak 2) was significantly decreased, but the formation of 4,4-dimethylzymosterol (peak 4) was rather slightly increased (Fig. V-4A). Moreover, in a preliminary experiment in which anti-cytochrome b_5 antibodies were added instead of palmitoyl-CoA, a radio-gas chromatogram similar to that shown in Fig. V-4A was obtained (data not shown). It is, therefore, evident that cytochrome b_5 participates in the 4-demethylation of 4,4-dimethylzymosterol, but not the 14α-demethylation of lanosterol.

Conclusion: All things considered, it can be concluded that both cytochrome b_5 and the cyanide-sensitive enzyme play essntial roles in the 4-demethylation of 4,4-dimethylzymosterol by yeast microsomes. By analogy with the fatty acyl-CoA desaturase system of yeast microsomes (12,68), it is highly likely that the cyanide-sensitive enzyme acts as the terminal monoxygenase reacting directly with the 4α-methyl group of the substrate and cytochrome b_5 is functional in the transfer of reducing equivalents derived from NADPH to the terminal enzyme. It is also likely that the electron transfer from NADPH to cytochrome b_5 is mediated by NADPH-cytochrome P-450 reductase, which has high cytochrome b_5 reducing activity (17).

Recently, a similar study was performed by Fukushima *et al.* (69) on the 4-demethylation reaction by rat liver microsomes and essentially the same results were obtained. So, the 4-demethylation reaction occurring in yeast and mammals is mediated by essentially the same enzyme system. However, very recently it was reported by Maitra *et al.* (70) that the 4-demethylation of 4,4-dimethylzymosterol was catalyzed only by a non-heme iron protein and no electron-transferring component was required for the reaction. This finding is inconsistent with ours and further work is needed to clarify the discrepancy.

TABLE V-I

EFFECT OF PALMITOYL-CoA ON $^{14}CO_2$ FORMATION FROM [^{14}C]LANOSTEROL BY YEAST MICROSOMES

The formation of $^{14}CO_2$ form [^{14}C]lanosterol (15,000 cpm) was mesured as described in MATERIALS AND METHODS, except that indicated concentrations of palmitoyl-CoA were added. Palmitoyl-CoA was added twice at time 0 and 15 min after initiation of the reaction, because the rate of palmitoyl-CoA desaturation was fairly high under these conditions.

Palmitoyl-CoA added (mM)	$^{14}CO_2$ formed (cpm)	Inhibition (%)
0	187	0
0.02	118	40.1
0.10	52	72.3

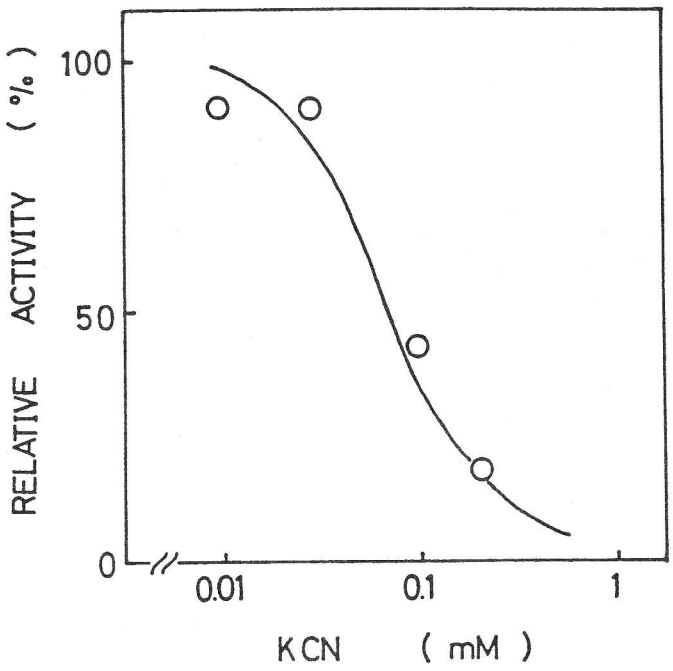


Fig. V-1. Inhibition of $^{14}CO_2$ formation from [^{14}C]lanosterol by KCN. The formation of $^{14}CO_2$ from [^{14}C]lanosterol (15,000 cpm) was mesured in the reaction mixture described in MATERIALS AND METHODS, except that indicated concentrations of KCN were added. In the absence of KCN 187 cpm of $^{14}CO_2$ were formed after 30 min incubation.

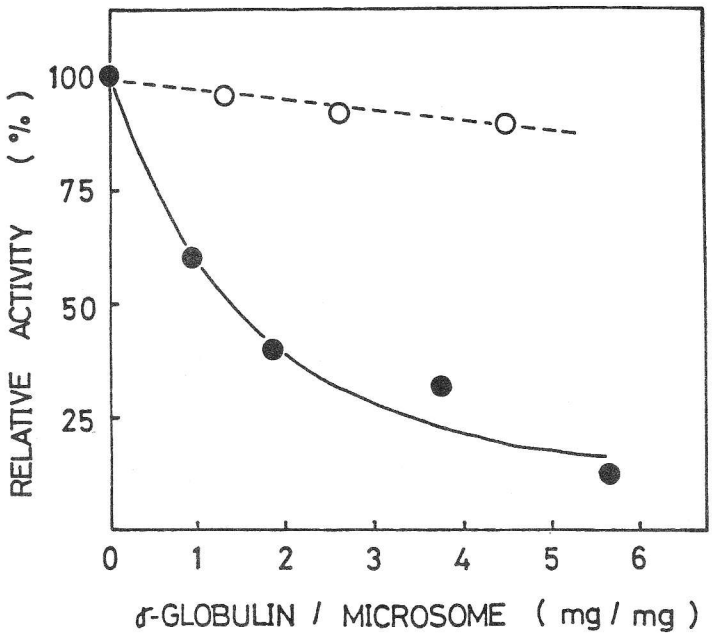


Fig. V-2. Inhibition of antimycin A-insensitive NADH-cytochrome c reductase activity by antibodies to yeast cytochrome b_5. Microsomes (0.54 mg protein) were preincubated in a spectrophotometer cuvette with the indicated amount of anti-cytochrome b_5 (—●—) or control (··o··) γ-globulin at 30°C for 15 min. Then, 20 μM cytochrome c, 0.5 mM KCN and 20 μg antimycin A were added to the cuvette. The reduction of cytochrome c was started by the addition of 0.15 mM NADH and followed spectrophotometrically at 550 nm.

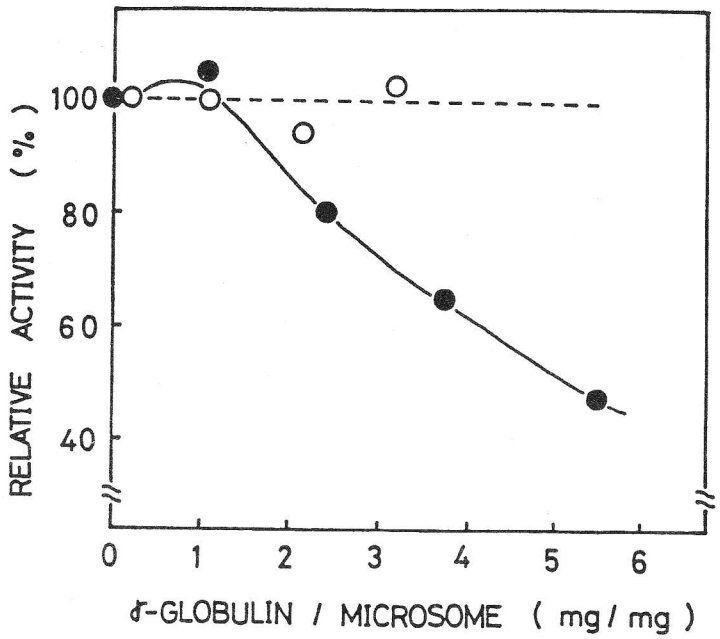


Fig. V-3. Effect of antibodies to yeast cytochrome $\underline{b}_5$ on the $^{14}CO_2$ formation from [^{14}C]lanosterol by yeast microsomes. Microsomes (4 mg protein) were preincubated with amount of anti-cytochrome $\underline{b}_5$ (-●-) or control (-○-) γ-globulin at 30°C for 15 min. The $^{14}CO_2$ from [^{14}C]lanosterol catalyzed by the pre= incubated microsomes was determined.

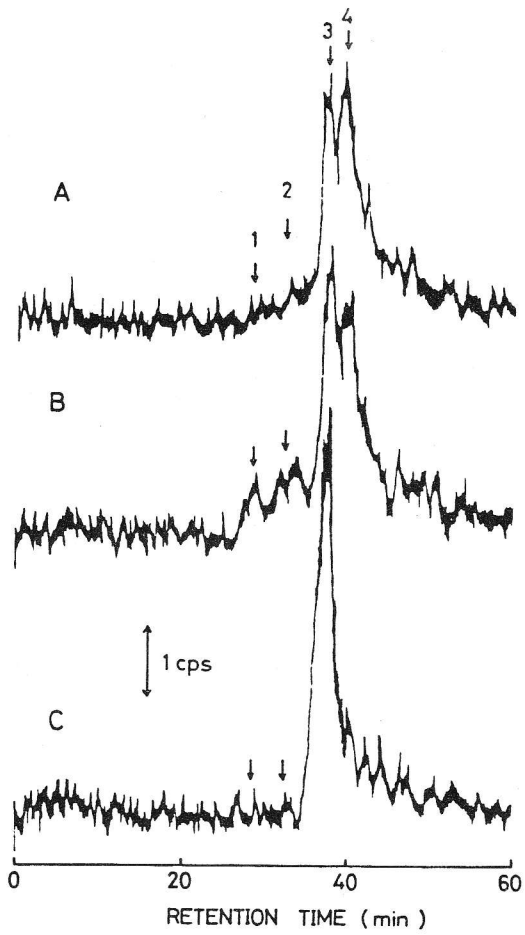


Fig. V-4. Radio-gas chromatograms of sterols foremd from [^{14}C]lanosterol in the presence (A) and absence (B) of palmitoyl-CoA and that extracted at zero time of incubation (C). A; [^{14}C]lanosterol was incubated with microsomes at 30°C for 30 min in the presence of 1.0 mM palmitoyl-CoA as described in Table V-I. Sterols were extracted and analyzed. B; Same as A, except that palmitoyl-CoA was omitted from the reaction mixture. C; The reaction mixture was the same as B, but sterols were extracted at zero time of incubation. In each experiment, extractes from five identical reaction mixtures were combined and subjected to radio-GLC.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSION

In this study, the author examined the roles of microsomal electron-transport system in lanosterol metabolism by yeast and the following findings were obtained. 1) A reconstituted system consisting of a cytochrome P-450 ($P\text{-}450_{14DM}$) and NADPH-cytochrome P-450 reductase both purified from yeast microsomes converted lanosterol to 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol in the presence of NADPH and molecular oxygen (Chapter II). 2) Microsomes from either semi-anaerobically or aerobically grown yeast cells metabolized lanosterol to 4,4-dimethylzymosterol and this metabolism was inhibited by rabbit antibodies raised against $P\text{-}450_{14DM}$ (Chapter III). 3) The second-derivative absorption spectrum of CO-reduced from of aerobically grown yeast microsomes gave spectral evidence for the occurrence of cytochrome P-450 in the microsomes which had been considered to contain no cytochrome P-450 (Chapter III). 4) Cyanide inhibited 4-demethylation of 4,4-dimethylzymosterol but not 14α-demethylation of lanosterol. 4-Demethylation of 4,4-dimethylzymosterol was inhibited by rabbit antibodies to yeast cytochrome $\underline{b}_5$ and also by palmitoyl-CoA (Chapter V). Based on these findings the roles of the microsomal electron-transport system in the lanosterol metabolism by yeast microsomes can be summarized as Fig. VI-1.

The 14α-demethylation of lanosterol to form 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol catalyzed by $P\text{-}450_{14DM}$ consists of three monooxygenation steps (see Scheme I-3), and the single cytochrome seems to catalyze all these reactions (Chapter II). Similar examples of cytochrome P-450-catalyzed C-C bond cleavage consisting of three oxygenation steps have been found in mammals. These are the side-chain cleavage of cholesterol to form pregnenolone catalyzed by $P\text{-}450_{SCC}$ (71,72) and fission between C-17 and C-20 of progesterone to form

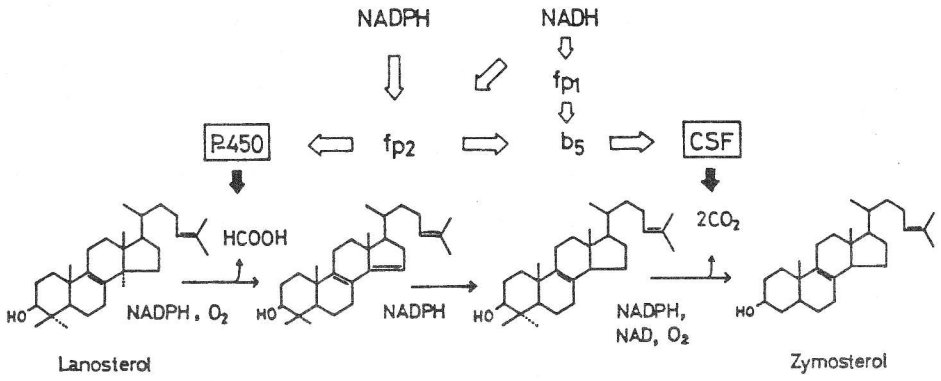


Fig. VI-1. The reoles of microsomal electron transport system in the conversion of lanosterol to zymosterol by yeast.

androstenedione catalyzed by a testicler microsomal cytochrome P-450 (73). In addition, the aromatization of androgens to estrogens, a reaction in which the C-19 methyl group is oxidatively removed and at the same time a double bond is introduced between C-1 and C-10, is exactly the same type of reacion as lanosterol 14α-demethylation and this reaction also seems to be catalyzed by a single cytochrome P-450 in placental microsomes (74-76). So, P-450$_{14DM}$ must be a type of cytochrome P-450 which is classified as a C-C lyase catalyzing oxidative cleavage of C-C bound through three oxigenation steps.

Recently, it was found by Hata et al. (65,66) that yeast microsomes contain another sterol-metabolizing cytochrome P-450 which catalyzes Δ^{22}-desaturation of ergosta-5,7-dien-3β-ol to form ergosterol. This indicates that multiplicity exists in sterol-metabolizing cytochrome P-450 of yeast. Regarding this, the action of buthiobate is noteworthy. As described in Chapter IV, buthiobate bound to P-450$_{14DM}$ with a high affinity and this binding induced a Type II

spectral change in the cytochrome, whereas the compound showed no effect on the Δ^{22}-desaturation. So, buthiobate seems to be a specific inhibitor for P-450$_{14DM}$ and may be an useful tool descriminating this cytochrome from other species of cytochrome P-450.

The 4-demethylation of 4-methylsterols by liver microsomes consists of oxygenation to form 4α-carboxylic acid derivative and the decarboxylation of the 4α-caboxylic acid derivatives (38,39) (Scheme I-4). Essentially the same mechanism seems to be functional in the 4-demethylation of 4,4-dimethylzymosterol by yeast microsomes. Since the latter step is known to proceed anaerobically (38,39), the enzyme system consisting of a cyanide-sensitive enzyme and cytochrome $\underline{b}_5$ is likely to be the oxygenase mediating the former step. Monooxygenase systems consisting of a cyanide-sensitive enzyme and cytochome $\underline{b}_5$ have been found in yeast microsomes other than the 4-demethylase described here. They are Δ^9-desaturase of palmitoyl-CoA (12,68) and Δ^5-desaturase of ergosta-7,22-dien-3β-ol (77). Although the cyanide-sensitive enzymes of these systems have not yet been isolated, different sensitivities of their activities to cyanide suggest multiplicity of the cyanide-sensitive enzymes.

Taken all together, the relationship between the microsomal electron-transport system and lipid metabolism occurring in yeast microsomes can generally be illustrated as Fig. VI-2. It is noteworthy that the terminal enzymes of the electron-transport system show great multiplicity and the multiplicity corresponds to their functional diversity. Similar diversity of the terminal enzymes is also observed in the hepatic microsomal system and the degree of diversity is more extensive than the yeast system. It can, therfore, be assumed that the microsomal electron-transport system has evolved by multiplying its terminal enzymes to adapt the increasing metabolic functions of hydrophobic substances including steroids, fatty acids, etc. Yeast is a primitive eukaryote.

So, fruits of this work may provide some important information concerning the comparative studies of mocrosomal electron-transport system to elucidate the evolutional background of this system.

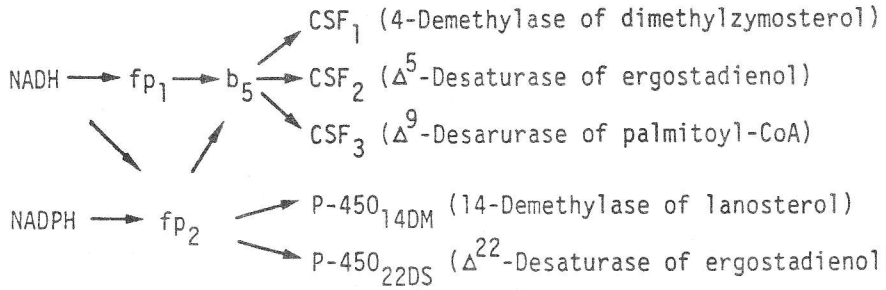


Fig. VI-2. Relationship between microsomal electron-transport system and lipid metabolism in yeast.

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