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INVOLVEMENT OF CYTOCHROME P-450 AND A CYANIDE-SENSITIVE ENZYME IN DIFFERENT STEPS OF LANOSTEROL DEMETHYLATION BY YEAST MICROSOMES

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SUMMARY: In the presence of NADPH, NAD^+, and molecular oxygen, microsomes prepared from Saccharomyces cerevisiae converted [1,7,15,22,26,30-^{14}C]lanosterol to 4,4-dimethylzymosterol, 4-methylzymosterol, and zymosterol, and this conversion was accompanied by the liberation of $^{14}CO_2$ derived from the methyl group (C-30) at the 4-position. The $^{14}CO_2$ formation was inhibited by antibodies to yeast cytochrome P-450 and by cyanide. Gas chromatographic evidence indicated that the antibodies inhibited the conversion of lanosterol to 4,4-dimethylzymosterol, whereas the demethylation of the latter to 4-methylzymosterol was sensitive to cyanide. It is concluded that cytochrome P-450 and a cyanide-sensitive enzyme are involved in the 14α- and 4-demethylations of lanosterol, respectively, by yeast microsomes.

The removal of three methyl groups of lanosterol (4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol) to form zymosterol (5α-cholesta-8,24-dien-3β-ol) is a step involved in the biosynthesis of cholesterol in animals and that of ergosterol in yeast. Alexander et al. (1) reported that this conversion in a cell-free system from Saccharomyces cervisiae is partially inhibited by CO and suggested the involvement of cytochrome P-450 in this process. Mitropoulos et al. (2) later showed that the 14α-methyl group of lanosterol is removed in the form of formic acid by the yeast cell-free system in a CO-sensitive reaction. More recently, Aoyama and Yoshida (3) presented evidence that lanosterol interacts with yeast cytochrome P-450 as a potential substrate in a reconstituted system consisting of purified cytochrome P-450 (4) and NADPH-cytochrome P-450 reductase (5). However, conclusive evidence for the func-

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tion of cytochrome P-450 in lanosterol demethylation has not yet been obtained. In this communication, we report that in yeast microsomes cytochrome P-450 is actually involved in the 14α-demethylation of lanosterol to 4,4-dimethylzymosterol (4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol) and that a cyanide-sensitive enzyme is functional in the conversion of 4,4-dimethylzymosterol to 4-methylzymosterol (4-methyl-5α-cholesta-8,24-dien-3β-ol).

MATERIALS AND METHODS. S. cerevisiae was grown semi-anaerobically as described (6) in a medium containing 2 % glucose, 0.5 % KH_2PO_4, 0.5 % yeast extract, and 0.5 % proteose peptone (pH 4). The cells were harvested at the late log phase and resuspended in 0.1 M potassium phosphate buffer (pH 6.2) containing 10 % glucose (25 g wet cells/liter). The suspension was shaken aerobically at 30° for 90 min to increase the sterol biosynthetic activity (7). The adapted cells (25 g wet weight) were suspended in 50 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 25 mg of glutathione and 175 mg of nicotinamide (8) and passed through a French pressure cell at a pressure of 400 kg/cm^2. The homogenate was centrifuged at 10,000 x g for 20 min, and the supernatant was again centrifuged at 78,000 x g for 90 min. The microsomal fraction thus precipitated was washed once with the same buffer and suspended (to 30 mg protein/ml) in 0.1 M potassium phosphate buffer (pH 7.5) containing 3 mM glutathione.

[1,7,15,22,26,30-^{14}C]Lanosterol (referred to as [^{14}C]lanosterol) was prepared enzymatically from [2-^{14}C]mevalonate by the method of Gibbons and Mitropoulos (9), and purified by thin-layer chromatography on $AgNO_3$-impregnated silica gel plates (17 %, w/w) using chloroform as solvent to remove dihydrolanosterol. The lanosterol band was eluted with methanol-benzene (1:1, v/v) and washed with 1 % NaCl and then with 1 % Na_2SO_3. Its content was determined as described by Stadtman (10)

Cytochrome P-450 was purified from yeast microsomes to a specific content of 13.2 nmoles/mg protein by the method of Yoshida et al. (4), and freed from the detergent, Emulgen 913, as follows. The preparation was applied to a CM-Sephadex C-50 column equilibrated with 20 mM potassium phosphate buffer (pH 7.25) containing 20 % glycerol. After washing the column with the same buffer until no absorption at 276 nm due to Emulgen 913 was detectable in the eluate, cytochrome P-450 was eluted with 0.2 M potassium phosphate buffer (pH 7.25) containing 20 % glycerol. The cytochrome was then dialyzed against 10 mM potassium phosphate buffer (pH 7.25) containing 0.9 % NaCl. A rabbit was immunized with the detergent-free cytochrome P-450 preparation essentially as described by Noshiro and Omura (11). The γ-globulin fraction of the antiserum, obtained by ammonium sulfate fractionation, was used as anti-cytochrome P-450 antibodies. The antibodies formed a single precipitin line with purified yeast cytochrome P-450 in Ouchterlony double diffusion test.

The formation of $^{14}CO_2$ from [^{14}C]lanosterol was studied in a reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.5), 1.5 mM NAD^+, 30-50 nmoles of [^{14}C]lanosterol (about 15,000 cpm), 0.05 % Tween 80, microsomes (10 mg of protein), and an NADPH-generating system consisting of 1.3 mM $NADP^+$, 15.6 mM DL-isocitrate, and 0.1 unit of isocitrate dehydrogenase in a final volume of 1.0 ml. Lanosterol was dissolved in 0.5 ml of diethyl ether plus 0.1 ml of 0.5 % Tween 80 in a test tube and the solution was stirred vigorously in a Thermo-Mixer under a stream of nitrogen gas to evaporate the ether completely. To the resulting lanosterol solution were added the other components of the reaction mixture and the tube was sealed with a rubber stopper to which a small cup containing 0.2 ml of Hyamine 10-X was hung. The reaction was started by placing the tube in a water bath kept at 37° and run for 60 min with constant shaking. The reaction was stopped by injecting 0.5 ml of 0.1 N HCl through the rubber stopper, and the mixture was shaken for 30 min to ensure the absorption of evolved CO_2 to Hyamine 10-X. The ^{14}C radioactivity of the Hyamine solution was counted in a Beckman LS-250 liquid scintillation spectrometer.

For analysis 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 (1.0 ml), and the mixture was saponified at 80° for 1 h. Sterols were extracted with petroleum ether, washed with water, and dried over anhydrous Na_2SO_4, and evaporated to a small volume under a stream of nitrogen gas. The radioactive sterol fraction was analyzed by radio-gas chromatography under the same conditions as described previously (12,13). Thus the analysis was carried out in a Shimadzu GC-5A gas chromatograph equipped with a thermo-conductive detector; the glass column (1.5 m x 4 mm) was packed with the stationery phases of Chromosorb W coated with 1.5 % (w/w) OV-17. Sterols were separated through this column at 240° 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 radioactivities were recorded with a Shimadzu SG-22 flow detector.

RESULTS AND DISCUSSION. In the presence of an NADPH-generating system, NAD^+, and molecular oxygen, microsomes from S. cerevisiae catalyzed the formation of $^{14}CO_2$ from [^{14}C]lanosterol, in which one of the two methyl groups at the 4-position (but not that at the 14α-position) was labeled. This activity (4-6 pmoles/min/mg protein) was proportional to the amount of microsomes up to 10 mg of protein in the reaction mixture (1.0 ml) and proceeded linearly at least for 60 min. Both NADPH and NAD^+ were obligatorily required for the activity. Under an atmosphere of $CO-O_2$ (80:20, v/v) the reaction was inhibited moderately (about 20 % inhibition), suggesting the involvement of a CO-binding component most probably cytochrome P-450. Actually, the $^{14}CO_2$ formation was found to be sensitive to antibodies to yeast cytochrome P-450; preincubation of yeast microsomes with the antibodies at a protein ratio of 1:1 resulted in more than 50 % inhibition, whereas the γ-globulin fraction prepared from an unimmunized rabbit caused no inhibition even when added at a globulin to microsomal protein ratio of 3:1. Although Alexander et al. (1) reported that the conversion of lanosterol to zymosterol by a cell-free yeast system was insensitive to cyanide, the $^{14}CO_2$ formation from [^{14}C]-lanosterol by yeast microsomes was very sensitive to cyanide; it was inhibited by about 90 % by 0.05 mM KCN and completely by 0.1 mM KCN. These findings indicated that both cytochrome P-450 and a cyanide-sensitive enzyme are functional in certain steps of the process leading to the formation of $^{14}CO_2$ from [^{14}C]lanosterol.

To obtain information concerning the origin of $^{14}CO_2$ and the steps catalyzed by cytochrome P-450 and the cyanide-sensitive enzyme, the metabolites formed from [^{14}C]lanosterol were analyzed by gas-liquid chromatography. To facilitate com-

parison of the retention times of the metabolites with those reported by Hatanaka et al. (12,13) for sterols formed from [^{14}C]mevalonate, the same chromatographic conditions as used by them were employed. As shown in Fig. 1A, four radioactive sterol peaks were detected and they were termed Peaks I through IV in the order of their retention times. Among them, Peak III which showed a retention times identical with that of lanosterol (Fig. 1B) was due to the unmetabolized substrate. As summarized in Table I, the retention times of Peaks I, II, and IV coincided closely with those of zymosterol, 4-methylzymosterol, and 4,4-dimethylzymosterol, respectively, which had been determined under the same chromatographic conditions (12,13). The formation of these metabolites can be explained only by assuming that the methyl group at the 14α-position of lanosterol was first removed followed by the removal of the two methyl groups at the 4-position by successive reactions. The pathway of lanosterol metabolism by yeast microsomes can, therefore, be expressed as follows:

Lanosterol (Peak III) → 4,4-dimethylzymosterol (Peak IV) → 4-methylzymosterol (Peak II) → zymosterol (Peak I)

This sequence indicates that the $^{14}CO_2$ formed under the conditions employed was derived from one of the methyl groups (C-30 of lanosterol) attached to the 4-position, since among the three methyl groups to be removed from [^{14}C]lanosterol only this one was labeled. From studies of sterols present in a sterol auxotroph of S. cerevisiae Trocha et al. (14) have suggested an alternate pathway in which the removal of one methyl group at the 4-position of lanosterol precedes the 14α-demethylation. However, this alternate pathway does not seem to be operative under the in vitro conditions employed in the present study.

As shown in Fig. 2A, the addition of anti-cytochrome P-450 antibodies to the reaction mixture (2 mg of antibodies per mg of microsomal protein) caused a marked change in the gas chromatographic pattern of sterol metabolites. Thus, the antibodies decreased the heights of Peaks I, II, and IV to almost insignificant levels, and caused intensification of Peak III. The addition of control γ-globulin, on the other hand, did not appreciably affect the chromatographic pattern, though

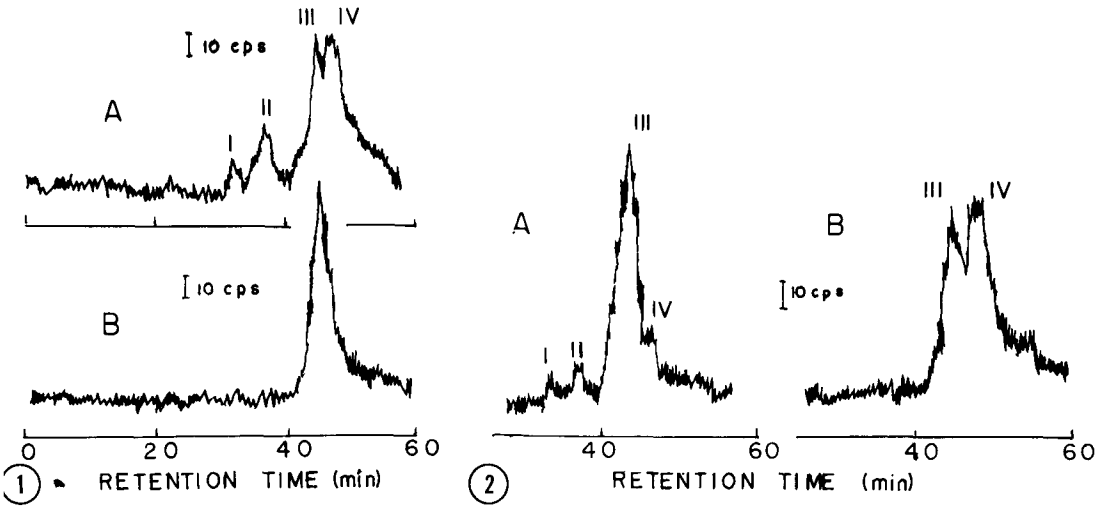

Fig. 1. Radio-gas chromatograms of metabolites formed from [^{14}C]lanosterol (A) and [^{14}C]lanosterol (B). Lanosterol was metabolized by yeast microsomes and sterol metabolites were extracted and assayed in the gas chromatograph as described in the "Materials and Methods" section, except that 30 nmoles (100,000 cpm) of [^{14}C]lanosterol was added to the incubation mixture.

Fig. 2. Effects of antibodies to yeast cytochrome P-450 (A) and cyanide (B) on radio-gas chromatogram of metabolites formed from [^{14}C]lanosterol. The experiments were carried out as described in Fig. 1, except that the antibodies (2mg/mg of microsomal protein) and 1 mM KCN were added in A and B, respectively.

TABLE I. Retention times and identification of Peaks I through IV in the radio-gas chromatogram shown in Fig. 1. The retention times of the four peaks were compared with those of sterol metabolites formed from [^{14}C]-mevalonate (12,13) and are expressed as relative values to that of squalene.

Peak	Relative Retention Time		Sterol
	This study	Refs. 12 & 13	
I	3.80	3.75	Zymosterol
II	4.28	4.10	4-Methylzymosterol
III	5.17	5.13	Lanosterol
IV	5.45	5.54	4,4-Dimethylzymosterol

slight intensification of Peak IV was noticed. It could be concluded that the antibodies inhibited the conversion of lanosterol (Peak III) to 4,4-dimethylzymosterol (Peak IV) and, therefore, to the other metabolites. When 1 mM KCN was in-

cluded in the reaction mixture, practically no formation of zymosterol (Peak I) and 4-methylzymosterol (Peak II) was observable, whereas 4,4-dimethylzymosterol (Peak IV) together with unmetabolized lanosterol (Peak III) were accumulated (Fig. 2B). It was thus clear that cyanide prevented the conversion of 4,4-dimethylzymosterol (Peak IV) to 4-methylzymosterol (Peak II). It could also be concluded that the methyl group removed in this conversion was the labeled one (C-30 of lanosterol), because 1 mM KCN could inhibit the $^{14}CO_2$ formation from [^{14}C]lanosterol completely. These observations led to the conclusion that in yeast microsomes cytochrome P-450 is involved in the oxidative removal of the 14α-methyl group from lanosterol and the cyanide-sensitive enzyme participation in the 4-demethylation of 4,4-dimethylzymosterol.

In confirmation of this conclusion, Aoyama and Yoshida [Aoyama, Y. and Yoshida Y., following paper] have recently shown that in the presence of NADPH and oxygen lanosterol can be converted to 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol by a reconstituted system consisting of purified yeast cytochrome P-450 and NADPH-cytochrome P-450 reductase. It seems that this product is reduced to 4,4-dimethylzymosterol before undergoing the cyanide-sensitive 4-demethylation. The cyanide-sensitive enzyme catalyzing the 4-demethylation is different from the cyanide-sensitive factor (desaturase) involved in palmitoyl-CoA desaturation by yeast microsomes in that the latter is much less sensitive to cyanide (15). It is of interest to note that the 4-demethylation of an artificial substrate, 4,4-dimethyl-5α-cholesta-7-en-3β-ol by rat liver microsomes in the presence of NADPH, NAD^+, and oxygen has also been reported to be strongly cyanide-sensitive (16). It is likely that closely similar enzymes are responsible for the sterol 4-demethylations in rat liver and yeast microsomes.

REFERENCES

1. Alexander, K.T.W., Mitropoulos, K.A., and Gibbons, G.F. (1974) *Biochem. Biophys. Res. Commun.* 60, 460-467
2. Mitropoulos, K.A., Gibbons, G.F., and Reeves, B.E.A. (1976) *Steroids* 27, 821-829
3. Aoyama, Y. and Yoshida, Y. (1978) *Biochem. Biophys. Res. Commun.* 82, 33-38

4. Yoshida, Y., Aoyama, Y., Kumaoka, H., and Kubota, S. (1977) *Biochem. Biophys. Res. Commun.* 77, 1005-1010
5. Aoyama, Y., Yoshida, Y., Kubota, S., Kumaoka, H., and Furumichi, A. (1978) *Arch. Biochem. Biophys.* 185, 362-369
6. Yoshida, Y., Kumaoka, H., and Sato, R. (1974) *J. Biochem.* 75, 1201-1210
7. Katsuki, H. and Bloch, K. (1967) *J. Biol. Chem.* 242, 222-227
8. Moore, J.T. and Gaylor, J.L. (1968) *Arch. Biochem. Biophys.* 124, 167-175
9. Gibbons, G.F. and Mitropoulos, K.A. (1973)*Biochem. J.* 132, 439-448
10. Stadtman, T.C. (1957) *Methods Enzymol.* 3, 392-393
11. Noshiro, M. and Omura, T. (1978) *J. Biochem.* 83, 61-77
12. Hatanaka, H., Ariga, N., and Katsuki, H. (1972) *Shishitsu Seikagaku Kenkyu (in Japanese)* 14, 211-216
13. Hatanaka, H., Ariga, N., Nagai, J., and Katsuki, H. (1974) *Biochem. Biophys. Res. Commun.* 60, 787-793
14. Troacha, P.J., Jane, S.T., and Sprinson, D.B. (1977) *Biochemistry* 16, 4721-4726
15. Tamura, Y., Yoshida, Y., Sato, R., and Kumaoka, H. (1976) *Arch. Biochem. Biophys.* 175, 284-294
16. Gaylor, J.L. and Mason, H.S. (1968) *J. Biol. Chem.* 243, 4966-4972