Vol. 85, No. 1, 1978
November 14, 1978

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 28-34

THE 14α-DEMETHYLATION OF LANOSTEROL BY A RECONSTITUTED CYTOCHROME P-450 SYSTEM FROM YEAST MICROSOMES

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Received August 4,1978

SUMMARY: Incubation of lanosterol (4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol) with a reconstituted system consisting of cytochrome P-450 and NADPH-cytochrome P-450 reductase, both purified from yeast microsomes, in the presence of NADPH and molecular oxygen resulted in the formation of a sterol product. This product showed a relative retention time to lanosterol of 1.10 on gas-liquid chromatography with a 1 % OV-17 column, and was identified as 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol by comparing its mass spectrum with that of lanosterol. It is concluded that the cytochrome P-450-containing monooxygenase system of yeast microsomes catalyzes the 14α-demethylation of lanosterol.

It is generally believed that the initial step of the biological conversion of lanosterol (4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol) to zymosterol (5α-cholesta-8,24-dien-3β-ol) is the removal of the 14α-methyl group (C-32 of lanosterol) (1). The chemical pathway of this demethylation has been fairly established as shown in Scheme 1 (2-12), but little is as yet known on the enzymes or enzyme systems involved in the process. Mitropoulos *et al.* (10) observed an inhibitory effect of CO on the formation of [^{14}C]formic acid from 24,25-dihydro[32-^{14}C]lanosterol both by rat liver microsomes and by a cell-free system from *Saccharomyces cerevisiae* and suggested the participation of cytochrome P-450 in this demethylation. In a previous paper (13) we also presented evidence that lanosterol interacts with the substrate site of purified yeast cytochrome P-450. In this communication, we now report that a reconstituted system consisting of cytochrome P-450 and NADPH-cytochrome P-450 reductase, both purified from *S. cerevisiae* microsomes, can actually catalyze the 14α-demethylation of lanosterol in the presence of NADPH and molecular oxygen.

0006-291X/78/0851-0028$01.00/0

MATERIALS AND METHODS

Enzyme Preparation: Cytochrome P-450 and NADPH-cytochrome P-450 reductase were purified from microsomes of anaerobically grown cells of *S. cerevisiae* as described by Yoshida *et al.* (14) and Aoyama *et al.* (15), respectively.

Lanosterol Preparation: The lanosterol preparation used (Serdary Research Laboratories Inc.) was found by gas-liquid chromatography to consist of 58 % of lanosterol and 42 % of 24,25-dihydrolanosterol. However, the presence of dihydrolanosterol had no effect on lanosterol metabolism. One mg of the lanosterol preparation containing 1.36 μmole of lanosterol and 7.5 mg of dilauroyl-L-3-phosphatidylcholine (Sigma Chemical Co.) were dispersed into 2.5 ml of 0.15 M KCl with the aid of sonic irradiation (30 KHz, 20 watts).

Lanosterol Metabolism by the Reconstituted System: Ten μl of cytochrome P-450 (0.36 nmole) in 10 mM potassium phosphate buffer (pH 7.0) containing 20 % glycerol and 0.2 % Emulgen 913 (a nonionic detergent), 100 μl of NADPH-cytochrome P-450 reductase (1.3 unit; for definition of unit, see Ref. 15) in 10 mM potassium phosphate buffer (pH 7.0) containing 0.2 M KCl, 0.5 % sodium cholate, and 1 mM EDTA, and 50 μl of the lanosterol dispersion (27.2 nmoles of lanosterol) were mixed in a reaction vessel at room temperature. The mixture was then diluted to 1.9 ml with 0.1 M potassum phosphate buffer (pH 7.2). The reaction was started by adding 0.1 ml of 3 mM NADPH and carried out at 30° for 30 min with constant shaking in air.

Analysis of the Metabolite: The reaction was stopped by adding 5 ml of 10 % (w/v) KOH in methanol, and the mixture was saponified at 80° for 10 min. Sterols were extracted with petroleum ether-diethyl ether (95:5, v/v) and the solvent was removed by evaporation under a stream of nitrogen gas. The residue was dissolved in 5 μl of 2-hexanone and analyzed in a Hitachi model 063 gas chromatograph equipped with a hydrogen-flame ionizing detector or in a Hitachi model RMU-6MG integrated gas chromatograph-mass spectrometer. Details of the procedures are given in the figure legends.

RESULTS AND DISCUSSION

The lanosterol dispersion was incubated aerobically with the reconstituted cytochrome P-450 system in the presence of NADPH and sterols extracted from the incubated mixture were analyzed by gas-liquid chromatography. As shown in Fig. 1A, three sterol peaks, termed Peaks 1, 2, and 3, were thus detected. Sterols extracted from the reaction mixture that had not been incubated, however, showed only Peaks 1 and 2 (Fig. 1B), which were identified as 24,25-dihydrolanosterol (the contaminant of the lanosterol preparation) and lanosterol, respectively, by compairing their retention times with those of the authentic samples. The intensity ratio of Peak 2 (lanosterol) to Peak 1 in the incubated mixture (Fig. 1A) was much smaller than that in the unincubated mixture (Fig. 1B), suggesting that the product (Peak 3) was formed at the expense of lanosterol. The metabolite formed showed a relative retention time to lanosterol of 1.10. Omission of either cytochrome P-450, the reductase, or NADPH from the reaction mixture as well as anaerobiosis resulted in practically no formation of Peak 3. The

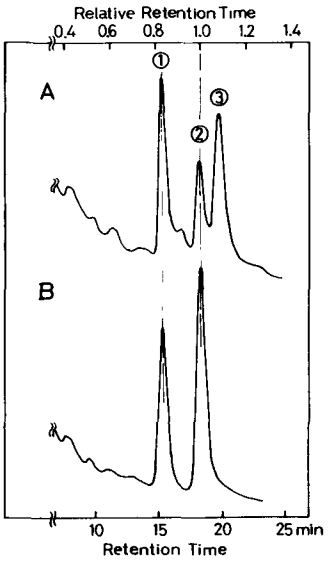


Fig. 1. Gas chromatograms of sterols extracted from the reaction mixture. A: Lanosterol was incubated at 30° for 30 min with the reconstituted system, and sterols were extracted and subjected to gas-liquid chromatography as described under MATERIALS AND METHODS. A coiled glass column (3 mm × 2 m) was packed with 1.0 % OV-17 on Chromosorv W (80-100 mesh, Wako Pure Chemical Industries) and N_2 was used as the carrier gas. The column temperature and the flow rate of carrier gas were fixed at 250° and 70 ml/min, respectively. B: Sterols extracted at time 0 from the reaction mixture were analyzed under the same conditions as above.

formation of the metabolite was also inhibited by about 80 % when the gas phase replaced by $CO-O_2$ (95:5, v/v), though no inhibition was observed when the gas pl was N_2-O_2 (95:5, v/v).

Fig. 2A shows the mass spectrum of the metabolite (Peak 3) formed from lano: As can be seen, the spectrum possesses nine characteristic peaks at m/e 410, 395, 3 328, 325, 313, 298, 283, and 246. The peak at m/e 410 can be assigned to the molecular peak of the compound, because it has the highest m/e value and the relative abundance of the other peaks was considerably increased when the ionizing energy of the mass spectrometer was increased (Fig. 2B). Furthermore, the peaks at m/e 395 a 377 can be identified as the fragments, $M-CH_3$ and $M-(CH_3+H_2O)$, respectively, which are species usually observable in the mass spectra of 3-hydroxysterols (16). It ca therefore, be concluded that the metabolite has a molecular weight of 410 and reta the 3β-hydroxy group of lanosterol. This in turn leads to another conclusion that one carbon and four hydrogen atoms must have been removed from lanosterol (M_r 426) upon its conversion to the metabolite. It is, therefore, likely that the reconstituted cytochrome P-450 system removed one methyl group from lanosterol and at the same time introduced an additional double bond in its ring system. As shown in

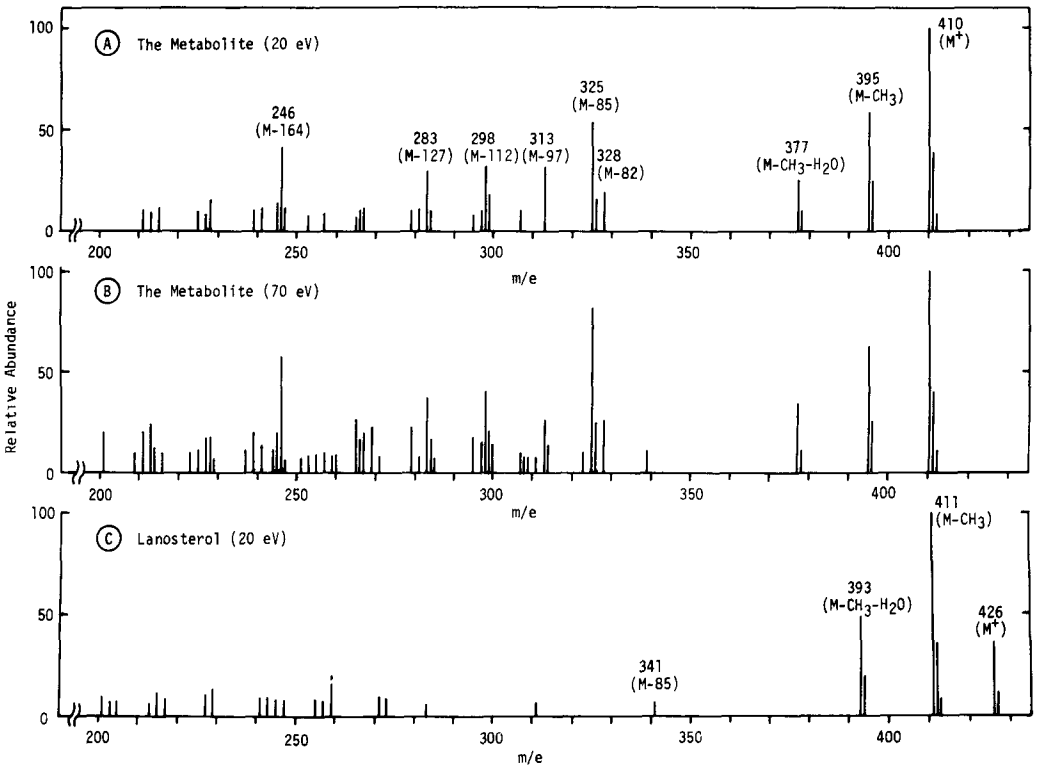
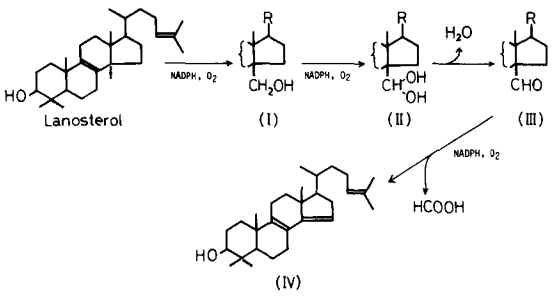


Fig. 2. Mass spectra of the metabolite and lanosterol. A: The same sample as used in the experiment of Fig. 1A was subjected to gas chromatography-mass spectrometry and the mass spectrum of Peak 3 in Fig. 1A was recorded. A coiled glass column (3 mm × 1 m) was packed with 1.0 % OV-17 on Chromosorv W (80-100 mesh) and He_2 was used as the carrier gas. The column temperature and the input pressure of carrier gas were fixed at 230° and 1.0 kg/cm^2, respectively. Scanning rate and ionization energy of the mass spectrometer were set at 100 mass/sec and 20 eV, respectively. B: The same experiment as A except ionization energy of the mass spectrometer was set at 70 eV. C: Mass spectrum of lanosterol (Peak 2 in Fig. 1) was recorded under the same conditions as A.

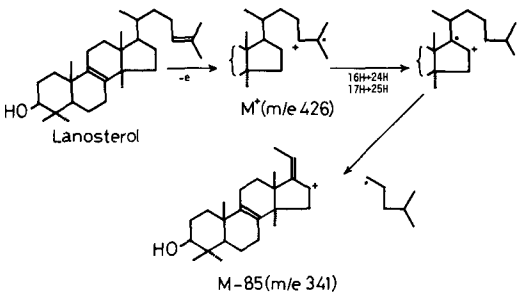
Scheme 1, such a compound has actually proposed as a lanosterol metabolite, *i.e.* 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol (Compound IV) (11,12).

The stractural identity of the metabolite with Compound IV can be examined by comparing its mass spectrum (Fig. 2A) with that of lanosterol (Fig. 2C). The base peak in the spectrum of lanosterol is that at m/e 411, which corresponds to an M-CH_3 fragment. The M-CH_3 fragment (m/e 395) can also be seen in the spectrum of the metabolite, but this peak is less intense than the molecular peak (m/e 410). The appearance of the intense M-CH_3 peak in the spectrum of lanosterol may be due to



Scheme 1. Postulated pathway for the 14α-demethylation of lanosterol (11,12).

the presence of 10β- and 14α-methyl groups, which are dissociable equally readily by the α-cleavage from $\Delta^{8(9)}$. If this is so, the less intense M-CH_3 peak in the spectrum of the metabolite suggests the absence of either 10β- or 14α-methyl group in the metabolite, because the $\Delta^{8(9)}$ should be remaining. In the mass spectrum of the metabolite, six conspicious fragments, M-82, M-85, M-97, M-112, M-127, and M-164 are observable, in addition to M^+, M-CH_3, and M-(CH_3+H_2O) which usually appear in the mass spectra of 3-hydroxysterols (16). From the m/e values M-82, M-85, and M-1 seem to have been formed by fragmentations at the side chain, and M-97 and M-127 ma be the demethylated ions derived from M-82 and M-112, respectively. M-164, on the other hand, may have been formed by cleavage of ring D accompanied by the loss of a angular methyl group. In the mass spectrum of lanosterol, on the other hand, no peaks corresponding to those fragments of the metabolite are observable, except for a very weak peak of M-85 (m/e 341), the formation of which can be explained by assuming the ionization at Δ^{24} as shown in Scheme 2. Therefore, the other fragments specific for the metabolite must have been formed by the ionization at the newly introduced double bond, which should be in ring D. Since the introduction of the double bond into lanosterol by the reconstituted cytochrome P-450 system should tak place in concert with the demethylation, it is expected that the introduced double bond is located between the α- and β-carbons from the metabolically removed methyl group. These considerations are, therefore, consistent with the view that the meta bolite has a stracture of 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol, which can b



Scheme 2. A possible mechanism for the formation of M-85 fragment (m/e 341) from lanosterol.

formed from lanosterol by removal of the 14α-methyl group and introduction of a new double bond between C-14 and C-15.

The results reported above thus indicate clearly that a reconstituted system consisting of yeast cytochrome P-450 and yeast NADPH-cytochrome P-450 reductase catalyzes the conversion of lanosterol to 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol in the presence of NADPH and molecular oxygen. As shown in Scheme 1, it is expected that this conversion involves three oxygenation steps, and the results obtained show that all these steps are catalyzed by the single species of cytochrome P-450. The participation of cytochrome P-450 in the 14α-demethylation of lanosterol in yeast is also consistent with the report by Mitropoulos *et al.* (10) that the 14α-methyl group of lanosterol is removed in the form of formic acid by a CO-sensitive enzyme system from *S. cerevisiae*. Furthermore, Ohba *et al.* [Ohba, M., Sato, R., Yoshida, Y., Nishino, K. and Katsuki, H., preceding paper] have recently shown that the conversion of lanosterol to 14-demethyllanosterol by yeast microsomes is inhibited by antibodies to yeast cytochrome P-450. It can be concluded therefore that the metabolism of lanosterol described here occurs also in intact yeast microsomes and the 14α-demethylation of lanosterol should proceed in three oxygenation steps as shown in Scheme 1, which are catalyzed by the single species of cytochrome P-450.

ACKNOWLEDGEMENT: We wish to thank Professor R. Sato of Osaka University for his interest and valuable suggestions made on this work.

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