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INVOLVEMENT OF CYTOCHROME P-450 IN Δ^{22}-DESATURATION IN ERGOSTEROL BIOSYNTHESIS OF YEAST

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SUMMARY: Δ^{22}-Desaturation of ergosta-5,7-dien-3β-ol to form ergosterol was studied with the microsomal fraction of Saccharomyces cerevisiae. NADPH and molecular oxygen were necessary for the reaction. The reaction was inhibited by carbon monoxide, metyrapone, cytochrome c, ferricyanide, menadione or p-hydroxymercuribenzoate but not inhibited by cyanide or azide. These results strongly suggested the involvement of cytochrome P-450-containing monooxygenase system in the reaction.

There remain some ambiguities in the studies on ergosterol biosynthesis in yeast. In the late stage of the biosynthesis, there are two desaturation reactions, namely cis-Δ^5- and trans-Δ^{22}-desaturations. We have already reported the involvement of cytochrome b_5 and a cyanide-sensitive enzyme in the Δ^5-desaturation (1). On the Δ^{22}-desaturation, however, scanty studies have been done. Goodwin and co-workers found that hydrogen atoms in different positions are eliminated depending on the kind of organism in the desaturation reaction (2). We previously suggested that molecular oxygen was involved in this reaction (3, 4) and that the reaction proceeded in the microsomal fraction of yeast (5). On the enzymatic mechanism of the reaction, however, no study has been made.

Nishikawa et al. (6) found that ergosta-5,7-dien-3β-ol ($\Delta^{5,7}$-C_{28}), rather than ergosterol, accumulated in the cells of Saccharomyces

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Abbreviations used: $\Delta^{5,7}$-C_{28}, ergosta-5,7-dien-3β-ol; GLC, gas-liquid chromatography.

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carlsbergensis when they were grown in the presence of thiamine. By feeding this yeast with L-[methyl-^{14}C]methionine, we could prepare [28-^{14}C]$\Delta^{5,7}$-C_{28}. The advantage of the use of $\Delta^{5,7}$-C_{28} as a substrate in the reaction in vitro is that the sterol is converted to only ergosterol.

The present communication describes the characterization of Δ^{22}-desaturation reaction of $\Delta^{5,7}$-C_{28} to form ergosterol with the microsomal fraction of Saccharomyces cerevisiae.

MATERIALS AND METHODS

[28-^{14}C]$\Delta^{5,7}$-C_{28} (specific radioactivity, 140 mCi/mol) was prepared as follows. Saccharomyces carlsbergensis 4228(ATCC 9080) was grown semi-anaerobically for 90 h at 30°C in the medium of Atkin et al. (7) with some modifications. Thiamine-HCl, cholesterol and Tween 80 were added to the medium at the final concentrations of 1 μg, 5 μg and 1 mg per ml, respectively. The cells (2 g in wet weight) were harvested and incubated aerobically with L-[methyl-^{14}C]methionine (7.5 μCi) with shaking for 15 h at 30°C in 200 ml of 0.1 M potassium phosphate buffer (pH 6.8) containing 4 g of glucose and 200 μg of thiamine-HCl. Radioactive sterols obtained from the cells after saponification were acetylated as reported (3). Sterol acetates thus obtained were separated by thin-layer chromatography on Silica gel G containing 20% $AgNO_3$ using benzene as a developing solvent. The mixture of acetates of ergosterol and $\Delta^{5,7}$-C_{28} (R_f 0.3-0.4) was eluted from the plate. The two sterol acetates were separated by GLC (column, 1.5 m x 3 mm, 3% SE-30; temperature, 205°C; carrier gas, N_2, 60 ml/min) with a Shimadzu GC-5A gas chromatograph. The derivative of $\Delta^{5,7}$-C_{28} emerged from the chromatograph (relative retention time using cholesteryl acetate as a standard, 1.42) was trapped by glass U-tubes cooled in ice and it was again subjected to a thin-layer chromatography. The purified derivative of $\Delta^{5,7}$-C_{28} was eluted from the plate and hydrolyzed with alkali.

Saccharomyces cerevisiae (ATCC 12341) was grown semi-anaerobically according to Katsuki and Bloch (8) and adapted aerobically as reported (3). The preparation of microsomal fraction followed by filtration through Sephadex G-50 was carried out according to Osumi et al. (1) except for the use of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM dithiothreitol instead of 0.1 M Tris-HCl buffer (pH 7.4). The gel-filtered microsomal fraction (6-8 mg of protein/ml) was used as an enzyme.

The standard reaction mixture contained, in a final volume of 1 ml, 16 nmol of [^{14}C]$\Delta^{5,7}$-C_{28}, (5,000 cpm), 0.5 μmol of NADPH, 0.1 μmol of dithiothreitol, 100 μmol of potassium phosphate buffer (pH 7.4) and the enzyme (4 mg of protein). The substrate was dispersed with the aid of 1 mg of Tween 20. Reaction was carried out with shaking for 1 h at 30°C. Then, the mixture was saponified, and unsaponifiable lipids were extracted as reported previously (9). After concentration of the extract, the lipids were subjected to a GLC as mentioned above. The fractions corresponding to ergosterol and $\Delta^{5,7}$-C_{28} emerged from the chromatograph were collected into glass U-tubes and each fraction was counted for its radioactivity. The enzyme activity was determined by measurement of radioactivity of ergosterol produced in the reaction for 1 h.

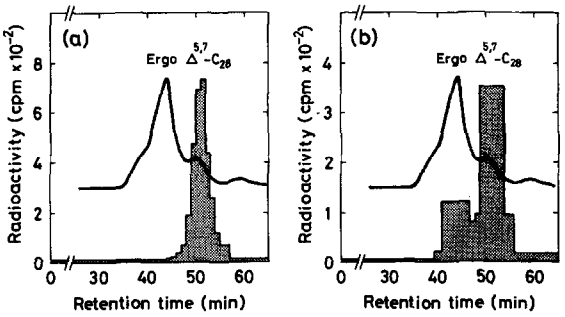

Fig. 1. ^{14}C-Incorporation from $[^{14}C]\Delta^{5,7}$-C_{28} into ergosterol. $[^{14}C]\Delta^{5,7}$-C_{28} was incubated with the gel-filtered microsomal fraction of yeast in the presence of NADPH. After saponification of the reaction mixture, sterols were extracted for GLC analysis. (a) and (b) show the chromatograms before and after the reaction, respectively. Solid line represents detector response of the chromatograph, indicating virtually that due to endogenous sterols since mass of radioactive sterols is negligibly small. Shadow part represents radioactivity. Ergo, ergosterol.

RESULTS

When $[^{14}C]\Delta^{5,7}$-C_{28} was incubated with the gel-filtered microsomal fraction of S. cerevisiae with shaking in the presence of NADPH, radioactivity was incorporated into ergosterol (Fig. 1). NADPH was necessary for the reaction, but NADH, $NADP^+$ or NAD^+ was less effective. The activity due to the presence of NADPH was not increased by the additional presence of NADH. For the reaction, molecular oxygen was also necessary. The activity of reaction under nitrogen was 77% that of reaction under air. Consumption of oxygen remaining in the reaction system by glucose (60 μmol) and glucose oxidase (EC 1.1.3.4) (17 units) lowered the activity to 23%.

The reaction was scarcely inhibited by 10 mM KCN or NaN_3 indicating the non-involvement of cyanide-sensitive enzyme in the reaction (Table 1).

The reaction was inhibited by carbon monoxide (Table 2) or metyrapone which is known to inhibit specifically cytochrome P-450 activity in adrenal mitochondria and that induced by phenobarbital in liver microsomes (10-12). The concentration of metyrapone necessary for 50% inhibition was 0.5 mM (Fig. 2). These results strongly suggest the involvement of cytochrome P-450 in the Δ^{22}-desaturation.

Table 1. Effects of cyanide and azide on Δ^{22}-desaturation activity. Reaction was carried out in the presence and absence of indicated compound. Figures in the parentheses represent relative activities taking the activity in "no addition" as a standard.

Expt.	Addition	Concentration (mM)	Activity (nmol)
1	None	—	2.97 (100)
	KCN	10	2.78 (94)
2	None	—	2.94 (100)
	NaN_3	10	3.01 (102)

The reaction was inhibited 97, 79, 100 and 85% by 0.5 mM cytochrome c, 5 mM potassium ferricyanide, 0.3 mM menadione and 0.2 mM p-hydroxymercuribenzoate, respectively. NADPH-cytochrome P-450 reductase was suggested to be involved in the reaction.

DISCUSSION

The results reported in the present communication strongly suggest that the Δ^{22}-desaturation in ergosterol synthesis of yeast has characteristics typical for cytochrome P-450-containing monooxygenase system in microsomes of mammalian liver. This is in contrast with Δ^{5}-desaturation in which cytochrome b_5 and a cyanide-sensitive enzyme were involved (1).

So far as known, no paper indicating the involvement of cytochrome P-450 in desaturation reaction seems to have appeared. A hydroxylated

Table 2. Effect of CO on Δ^{22}-desaturation activity. Reaction was carried out under indicated gas mixture in Thunberg tubes.

Gas phase (by vol.)	Activity (nmol)
N_2-O_2 (94:6)	2.44 (100)
CO-O_2 (94:6)	1.29 (53)

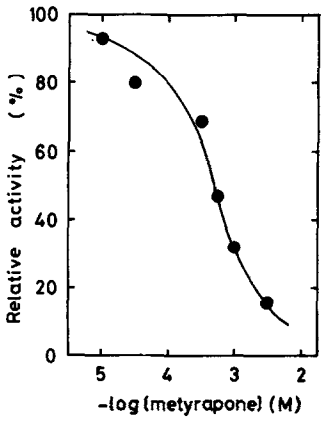

Fig. 2. Effects of various concentrations of metyrapone on Δ^{22}-desaturation activity.

compound may be formed as an intermediate in the reaction followed by subsequent dehydration of it. However, no such an intermediate has been detected. The Δ^{22}-desaturation seems to be a unique reaction in which cytochrome P-450 is involved.

Further study is now in progress on the mechanism of the reaction.

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