A POSSIBLE ROLE FOR CYTOCHROME P-450 DURING THE BIOSYNTHESIS OF ZYMOSTEROL FROM LANOSTEROL BY *SACCHAROMYCES CEREVISIAE*

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SUMMARY

A cell-free system has been obtained from *Saccharomyces cerevisiae* which is capable of efficiently converting lanosterol[1] to a mixture of 4-demethyl sterols, quantitatively the most important identifiable component of which was zymosterol. Little or no ergosterol was synthesized. In the presence of carbon monoxide, the rate of zymosterol biosynthesis from lanosterol was decreased by 57 % compared with that observed in control incubations and the amount of unmetabolized lanosterol was greater. Mitochondrial electron transport inhibitors such as cyanide and antimycin A had no effect on the overall rate of 4-demethyl sterol biosynthesis from lanosterol nor on the degree of inhibition by carbon monoxide.

Cytochrome P-450 has been reported to occur in various species of fungi (1-7) and has been solubilized from microsomal preparations of the yeasts *Saccharomyces cerevisiae* (5) and *Candida tropicalis* (6, 7). In the latter species, the solubilized haemoprotein has been shown to catalyze the ω-oxidation of exogenously added fatty acids (6, 7), and the hydroxylation of exogenously added hydrocarbons and drugs (6). However, at present there is no evidence for the rôle of cytochrome P-450 in an established metabolic sequence resulting in the biosynthesis of a natural product of *S. cerevisiae*. In mammalian liver, cytochrome P-450 is involved in the oxidative metabolism of lanosterol (8), a cholesterol precursor, and, in view of the close similarity between the biosynthesis of ergosterol in fungi and cholesterol in higher animals (9), it was considered possible that the haemoprotein could

[1]Systematically, lanosterol is 5α-lanosta-8,24-dien-3β-ol, zymosterol is 5α-cholesta-8,24-dien-3β-ol, ergosterol is ergosta-5,7,22-trien-3β-ol, and 14-demethyllanosterol is 4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol.

fulfil a similar function during yeast sterol synthesis, a process during which the intermediacy of lanosterol is well established (9). The present investigation provides evidence that a carbon-monoxide inhibitable enzyme is required for the oxidative conversion of lanosterol to zymosterol during ergosterol biosynthesis in Saccharomyces cerevisiae.

MATERIALS AND METHODS

Saccharomyces cerevisiae (LK_2G_{12}) was grown semi-anaerobically in a nutrient medium followed by a period of aerobic growth in the presence of a high glucose concentration, according to the method of Katsuki and Bloch (10). The cells were disrupted by the method of Klein (11) and the resulting suspension was centrifuged at 2,000 g for 10 min. The supernatant was filtered through glass wool, recentrifuged at 2,000 g, filtered again, and finally centrifuged at 26,000 g for 30 min. The floating fat layer was removed and the heavy particulate material from this centrifugation was resuspended in the supernatant. This suspension was used as the source of enzyme. Incubation of cell-free systems with [^{14}C]lanosterol in the presence of gas mixtures has been described previously (8, 12, 13). After incubation, radioactive lipid precursors of ergosterol were separated by t.l.c. on silicagel H using chloroform as the mobile phase (System 1). Further purification was effected by acetylation of the appropriate sterol fraction followed by t.l.c. on silver nitrate-impregnated silicagel H using a mixture of toluene and hexane (70:30, v/v) (System 2) as the mobile phase, or on silver nitrate-impregnated alumina (14) with a mixture of toluene and hexane (25:75, v/v) as the developing solvent (System 3). After each type of chromatography, radioactive zones were located by radioautography.

[1,7,15,22,26,30-$^{14}C_6$]lanosterol (referred to as [^{14}C]lanosterol) was prepared as described previously (13). Zymosterol was extracted from "active" bakers' yeast (Distillers Co. Ltd., Morden, Surrey, U.K.).

Protein was assayed by the method of Cleland and Slater (15) and 4-demethyl sterols were purified through the digitonide by the procedure of Goad and Goodwin (16)

RESULTS

Effect of carbon monoxide on 4-demethyl sterol synthesis from [^{14}C]lanosterol. [^{14}C]Lanosterol was incubated with the cell-free system in the presence or absence of CO. Separation of the metabolic products showed that the presence of CO in the gas phase resulted in a decrease in the rate of synthesis of the 4-demethyl sterol mixture (Table 1) and the radioactivity of

Table 1. Effect of CO on the incorporation of radioactivity from [^{14}C]lanosterol into zymosterol by a *S. cerevisiae* subcellular fraction

[^{14}C]Lanosterol (171800 d.p.m.) was incubated with an enzyme system (2.0 ml; 5.3 mg protein/ml) consisting of the recombined particulate and supernatant fractions, in the presence of gas phases consisting either of N_2 + O_2 (90:10, v/v) or CO + O_2 (90:10, v/v). The incubation mixture contained 20.4 mM GSH, 6.9 mM ATP, 2.9 mM NAD^+, 0.7 mM CoA, 2.0 mM fructose-1,6-diphosphate, 1.0 mM $MgSO_4$, 2.0 mM $MnSO_4$, 4.2 mM L-methionine, 2.6 mM $NADP^+$, 10.3 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase (1 μmole of NADPH generated/min) and 0.1 M potassium phosphate buffer (pH 7.3) in a total volume of 2.4 ml. After 3 h at 30° C, each incubation was terminated with ethanol (4.0 ml) containing "carrier" lanosterol and ergosterol (2.0 mg of each). After alkaline hydrolysis[1], the 4,4-dimethyl sterol fraction was separated from that containing the 4-demethyl sterols by t.l.c. of the extracted lipid in System 1 and a portion of each fraction was removed for counting of radioactivity. The fraction containing the acetate of zymosterol was obtained by acetylation of the 4-demethyl sterol fraction (after the addition of carrier zymosterol (1.0 mg)) followed by t.l.c. in System 2. Each incubation was conducted in duplicate and each reported figure is the average of the two values. Figures in parentheses represent the amount of radioactivity associated with each fraction as a percentage of the radioactivity incubated.

Gas phase	Radioactivity (d.p.m.) associated with			Relative effect of CO on zymosterol synthesis
	4,4-Dimethyl sterols	4-Demethyl sterols	Zymosteryl acetate	
N_2 + O_2	59735 (34.8)	16438 (9.6)	5100 (3.0)	100
CO + O_2	99767 (58.1)	12770 (7.4)	3689 (2.2)	72.3

[1]The pH of the hydrolyzed solution was adjusted to pH 7.0 by the addition of 2N HCl (19) prior to extraction with ether. The residual aqueous phase was reduced in volume by evaporation at reduced pressure and subjected to methanolic alkaline pyrogallol hydrolysis (20). However, after extraction of the hydrolysate with ether, no further radioactivity was recovered.

the 4,4-dimethyl sterol fraction (containing predominantly unmetabolized [^{14}C]lanosterol) was much greater. Chromatographic separation of the individual components of the acetylated 4-demethyl sterol fraction showed that, in each case, the major identifiable component had the chromatographic properties of zymosteryl acetate and that the radioactivity associated with this compound was greater in those incubations conducted in the absence of carbon monoxide. During this chromatography, a large proportion of the radioactivity of each biosynthetic 4-demethyl sterol fraction remained at the origin of the plate. This indicated the presence of a labile 4-demethyl sterol component and the *in vitro* formation of a sterol of this type has been reported previously (10). In accordance with a previous report (10) we were unable to detect any significant incorporation of radioactivity into ergosterol. This was the case regardless of the nature of the gas phase. Zymosterol, therefore, was the major 4-demethyl sterol product of lanosterol metabolism in the present system. Addition of carrier material to the fractions containing zymosteryl acetate followed by recrystallization to constant specific radioactivity showed that in both types of incubation 80% of the initial observed radioactivity was associated with zymosteryl acetate.

Effect of inhibitors of mitochondrial electron transport on the extent of CO inhibition of 4-demethyl sterol synthesis. The heavy particulate fraction which was required for efficient 4-demethyl sterol synthesis contains mitochondrial (11) or promitochondrial (17) particles. In view of the reported stimulatory effect of mitochondrial electron transport inhibitors on the rate of sterol synthesis during the aeration of yeast *in vivo* (18) and the fact that CO is an efficient inhibitor of mitochondrial cytochrome oxidase activity, it was considered necessary to eliminate any ambiguity which may arise from an indirect effect of CO in its capacity as an inhibitor of mitochondrial electron transport, on the rate of 4-demethyl sterol synthesis. Any effect due to a change in the rate of mitochondrial electron transport in the present system was therefore removed by the addition of either cyanide or antimycin A to the cell-free system before incubation with [^{14}C]lanosterol in

the presence of either $N_2 + O_2$ or $CO + O_2$. Similar incubations were conducted in the presence of the gas phases but in the absence of any additional electron transport inhibitor. In those incubations conducted in the presence of carbon monoxide, regardless of the presence or absence of antimycin and cyanide, the amount of radioactivity associated with the 4-demethyl sterol fraction was much less than that observed in corresponding incubations conducted in the presence of $N_2 + O_2$ and there was a much greater amount of radioactivity associated with the 4,4-dimethyl sterol fraction (Table 2). In addition, the presence of antimycin or cyanide had no effect either on the incorporation of [^{14}C]lanosterol into 4-demethyl sterols, or on the extent of inhibition of this process by carbon monoxide. Isolation of zymosterol (as the 3β-acetate) from each of the 4-demethyl sterol fractions showed that this material was biosynthesized to a much smaller extent during incubations conducted in the presence of CO (Table 2). There was no radioactivity corresponding to ergosterol (isolated as the 3β-acetate) in any of the incubation mixtures, although in all cases, after chromatography of the acetylated 4-demethyl sterol mixture, a considerable amount of radioactivity remained at the origin of the chromatoplate.

DISCUSSION

The existence of cytochrome P-450 in some species of yeasts has been reported (3-7) and, although a preparation of the haemoprotein solubilized from Candida tropicalis is involved in the oxidative metabolism of exogenous substrates (6, 7), a rôle for cytochrome P-450 in endogenous metabolic pathways has yet to be established. The present work has shown that CO has a direct inhibitory effect on the rate of 4-demethyl sterol synthesis from lanosterol in cell-free systems of S. cerevisiae and that the rate of biosynthesis of an identifiable component of this mixture, zymosterol, is considerably decreased. In addition, CO resulted in a large decrease in the rate of metabolism of lanosterol itself, as judged by the amount of unchanged [^{14}C]-lanosterol recovered at the end of the incubations.

Table 2. Effect of mitochondrial electron transport inhibitors on the rate of 4-demethyl sterol synthesis from [^{14}C]lanosterol in the presence and absence of CO

[^{14}C]Lanosterol (194400 d.p.m.) was incubated with an enzyme system (2.0 ml; 10.0 mg protein/ml) consisting of the recombined particulate and supernatant fractions, in the presence of gas phases consisting of N_2 + O_2 (90:10, v/v) or CO + O_2 (90:10, v/v). The concentration of cofactors was the same as that given in Table 1. Similar incubations were conducted with [^{14}C]lanosterol in the presence of either CO + O_2 or N_2 + O_2, using the same cofactors, and in addition, either 0.83 mM KCN or 8 μg of antimycin A. The final volume of each incubation mixture was 2.4 ml. After 2 h at 30° C, incubations were terminated with ethanol (4.0 ml) containing "carrier" ergosterol and lanosterol (2.0 mg of each). After acid and alkaline hydrolysis, the 4,4-dimethyl sterol fraction was separated from that containing the 4-demethyl sterol by t.l.c. of the extracted lipid (System 1). After addition of carrier zymosterol (1.0 mg), radioactive zymosteryl acetate was isolated by acetylation of each 4-demethyl sterol mixture followed by t.l.c. in System 2.

Gas phase	Mitochondrial E.T. inhibitor	Radioactivity (d.p.m.) associated with			Relative effect of CO on the synthesis of	
		4,4-Dimethyl[1] sterols	4-Demethyl sterols	Zymosteryl acetate	4-Demethyl sterols	Zymosterol
N_2 + O_2	-	28900 (14.8)	29600 (15.2)	8680 (4.5)	100	100
CO + O_2	-	95900 (49.3)	12600 (6.5)	3770 (1.9)	42.6	43.4
N_2 + O_2	Antimycin A	27200 (14.0)	27300 (14.0)	13120 (6.7)	100	100
CO + O_2	Antimycin A	95000 (48.9)	11600 (6.0)	4700 (2.4)	42.5	35.8
N_2 + O_2	KCN	25900 (13.3)	27700 (14.3)	10970 (5.6)	100	100
CO + O_2	KCN	94600 (48.7)	11100 (5.7)	3070 (1.6)	40.1	28.0

[1]In all incubations conducted in the presence of CO, [^{14}C]lanosterol contributed 83-88 % of the total radioactivity of the 4,4-dimethyl sterol fraction, as estimated by t.l.c. of the acetylated fraction in System 3. The corresponding values for incubations conducted in the presence of N_2 + O_2 were 64-69 %.

In all incubations in which there was a higher rate of [^{14}C]lanosterol metabolism (i.e. in the presence of N_2 + O_2), the rate of disappearance of lanosterol was not fully compensated for by the amounts of radioactivity associated with the 4α-methyl and 4-demethyl sterol fractions. This discrepancy was not due to the formation of mannan-bound water-soluble sterols (19) as those sterols would have been released during methanolic pyrogallol saponification of the aqueous phase after ether extraction of the hydrolyzed incubation contents (20). In these cases, the difference was probably due either to further metabolism of the 4-demethyl sterols to acidic products (21) or to the formation of unstable sterol precursors of ergosterol which were degraded during the extraction and chromatographic procedures (10). Whichever may be the case, calculation of the extent of CO inhibition on the basis of the amount of 4-demethyl sterol synthesized may be an underestimate and perhaps a more valid approach would be the measurement of unmetabolized lanosterol, a method which would result in a larger estimate of the degree of CO inhibition. 14-Demethyllanosterol has been shown to be present in yeasts (22, 23) and it is probable that the first step in the biosynthesis of zymosterol from lanosterol involves the oxidative removal of the 14α-methyl group. The decreased rate of metabolism of lanosterol therefore indicates that a CO-inhibitable enzyme is involved during this process. This situation, therefore, is very similar to that which arises during cholesterol biosynthesis in mammalian liver, a process which has been shown to involve cytochrome P-450 (8). In view of this, and of the close similarity between the overall processes of cholesterol and ergosterol synthesis, it is tentatively suggested that this CO-inhibitable lanosterol 14α-methyl demethylase involves cytochrome P-450 and that one of the metabolic rôles of the haemoprotein in yeast is its involvement in the latter stages of ergosterol biosynthesis.

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