TARGET OF INHIBITION BY THE ANTI-LIPOGENIC ANTIBIOTIC CERULENIN OF STEROL SYNTHESIS IN YEAST

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Received March 4,1974

SUMMARY. The antibiotic cerulenin inhibited the incorporation of ^{14}C-acetyl-CoA by 67% at a concentration of 9 x 10^{-6} M but not that of ^{14}C-HMG-CoA into the non-saponifiable fraction in a cell-free extract of *Saccharomyces cerevisiae*. Cerulenin markedly inhibited the activity of partially purified HMG-CoA synthase. No inhibition of acetoacetyl-CoA thiolase activity was observed in the same preparation of HMG-CoA synthase. Therefore, cerulenin inhibition of overall sterol synthesis may be accounted for by the specific inhibition of HMG-CoA synthase activity.

INTRODUCTION. It has been reported from this laboratory that cerulenin, an antibiotic of the structure (2S)(3R)2,3-epoxy-4-oxo-7,10-dodecadienoyl amide (1-3), reduced overall synthesis of fatty acids and digitonin-precipitable sterols both *in vivo* and *in vitro* in yeast (4, 5). The mechanism of cerulenin inhibition of fatty acid synthesis has been precisely studied under the collaboration of groups of Bloch, Vagelos, and this laboratory (6, 7). Cerulenin specifically blocks the activity of β-ketoacyl-ACP synthetase by binding irreversibly at the molar ratio 1:1 (7). However, the mechanism of cerulenin inhibition of sterol synthesis has remained obscure. Although Nomura et al (5) have reported that cerulenin inhibited ^{14}C-acetyl-CoA incorporation but not ^{14}C-mevalonate incorporation into NSF in a cell-free extract of yeast, still 3 steps have remained to be clarified, namely the steps catalyzed by acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase which has been

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Abbreviations: HMG-CoA = β-Hydroxy-β-methylglutaryl Coenzyme-A
NSF = non-saponifiable fraction
ACP = acyl carrier protein

known as a rate-limiting enzyme of overall sterol synthesis in rat liver (8). In the present paper, we report the rather specific inhibition of HMG-CoA synthase activity by cerulenin.

MATERIALS AND METHODS. *Saccharomyces cerevisiae* ATCC 12341 was grown in a semi-synthetic medium and cell free extract was prepared as reported by Kawaguchi (9). Reaction mixtures and incubation conditions are given in the legends of Figures and a Table. The reaction mixture was saponified with 15% KOH in 80% methanol at 60°C for 1 hr. Non-saponifiable fraction (NSF) was isolated by petroleum ether extraction. The radioactivity was measured in a liquid scintillation counter. Acetoacetyl-CoA thiolase and HMG-CoA synthase were partially co-purified from bakers yeast (generous gift from Oriental Yeast Co. Ltd., Tokyo) according to the method of Ferguson and Rudney (10) up to the step of 80% saturated $(NH_4)_2SO_4$ precipitation after the treatment by protamine sulfate. Enzyme activity was assayed spectrophotometrically at room temperature. Albumin in the original assay mixture has been omitted since it improved enzyme stability only marginally, and slightly disturbed cerulenin inhibition of HMG-CoA synthase activity. Specific activities were 13.9 and 9.7 units/mg for acetoacetyl-CoA thiolase and HMG-CoA synthase respectively. Acetyl-CoA and acetoacetyl-CoA were purchased from Sigma Co. Ltd. (St. Louis). 1-^{14}C-acetyl-CoA and 3-^{14}C-3-hydroxy-3-methyl glutaric acid were purchased from New England Nuclear (Boston), 3-^{14}C-HMG-CoA was synthesized by the methods of Goldfarb and Pitot (11). 2-^{14}C-mevalonic acid dibenzyl-ethylene-diamine salt was purchased from Daiichi Chemicals Co. (Tokyo).

RESULTS. In a cell free extract of *S. cerevisiae*, cerulenin inhibited ^{14}C-acetyl-CoA incorporation into NSF by 67% at 9×10^{-6} M (2 μg/ml), as shown in Fig. 1. This inhibition is sufficient to account for the inhibition of overall sterol synthesis *in vivo* (4). Contrarily, the incorporation of ^{14}C-HMG-CoA or ^{14}C-mevalonate was not reduced up to concentrations of 2.3 x

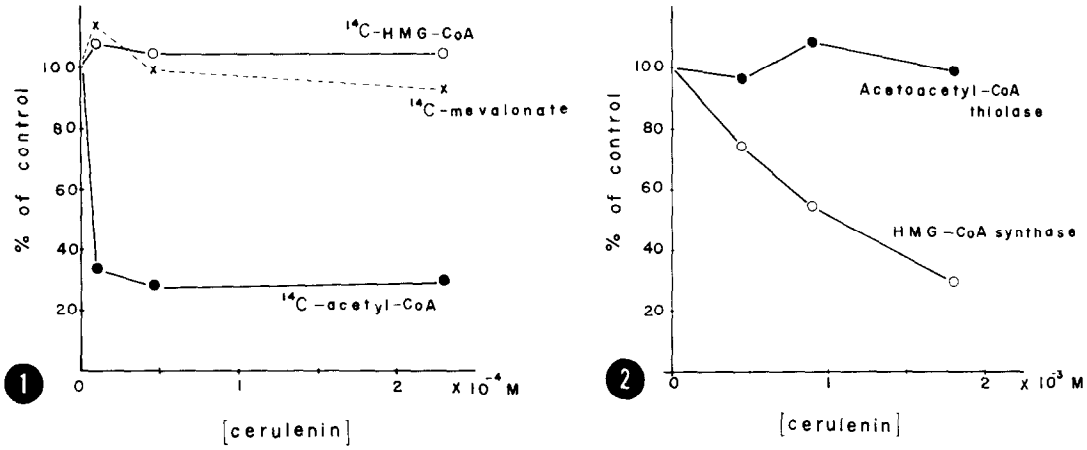

Fig. 1. Effect of cerulenin on the incorporation of ^{14}C-precursors of sterol synthesis into the non-saponifiable fraction in cell free extracts of *S. cerevisiae*.

The reaction mixture contained 80 µmoles of phosphate buffer, pH 7.0, 5 µmoles of ATP, 0.1 µmole of CoA, 1 µmole of NADP, 2 µmoles of GSH, 10 µmoles of glucose-6-phosphate, 2 µmoles of $MgSO_4$, 1 µmole of $MnSO_4$, 0.3 ml of yeast cell free extract (20 mg of protein), and cerulenin in a total volume of 0.9 ml. After pre-incubation for 15 min, 0.1 µCi of 3-^{14}C-HMG-CoA (0.5 µmoles), 2-^{14}C-mevalonate (0.046 µmoles), or 1-^{14}C-acetyl-CoA (0.002 µmoles) in 0.1 ml aqueous solution was added and further incubated for 15 min at 37°C. The control value of incorporation was 7150 cpm for ^{14}C-HMG-CoA, 8736 cpm for ^{14}C-mevalonate and 3580 cpm for ^{14}C-acetyl-CoA.

Fig. 2. Effect of cerulenin on the activity of acetoacetyl-CoA thiolase and HMG-CoA synthase.

The reaction mixture contained 100 µmoles of tris-HCl buffer (pH 8.1), 0.3 µmole of acetoacetyl-CoA, 0.2 µmoles of CoA or acetyl-CoA, cerulenin and enzyme (0.230 mg for acetoacetyl-CoA thiolase assay, 0.345 mg for HMG-CoA synthase assay) in a total volume of 1 ml. Enzymes were pre-incubated for 30 min with cerulenin at room temperature (25° ± 0.5°C). CoA or acetyl-CoA was added after 5 min-trace of base line of the absorbance of acetoacetyl-CoA at 310 nm in a cuvette with a 1-cm light pass. Initial velocity of the absorbance decrease in controls were 0.032 and 0.028 for acetoacetyl-CoA thiolase and HMG-CoA synthase respectively.

10^{-4} M. This fact suggests that cerulenin has no effect on HMG-CoA reductase activity and subsequent steps of sterol synthesis. Therefore, the two catalytic steps, acetyl-CoA to acetoacetyl-CoA then to HMG-CoA, must be affected by cerulenin. The two enzymes for these steps, acetoacetyl-CoA thiolase and HMG-CoA synthase, have been reported to have closely similar physical properties and to be easily assayable separately by utilizing aceto-

Table 1. Effect of preincubation on cerulenin inhibition of HMG-CoA synthase activity.

preincubation time	A_{310} decrease per min	
	control	cerulenin
0 min	0.032	0.032
8	0.033	0.026
30	0.028	0.008

The assay conditions are fundamentally equal to those described in Fig. 2. Cerulenin concentration was 1.8×10^{-3} M.

acetyl-CoA as a common substrate. These enzymes were co-purified according to the method of Ferguson and Rudney (9) up to the step of protamine sulfate treatment and concentrated by 80% saturated ammonium sulfate precipitation.

As shown in Fig. 2, cerulenin had no effect on acetoacetyl-CoA thiolase activity at a concentration of 1.8×10^{-3} M, but markedly inhibited HMG-CoA synthase activity. However, the cerulenin concentration required for 50% inhibition of the enzyme activity was 1.1×10^{-3} M which was far higher than that required to inhibit incorporation of ^{14}C-acetyl-CoA into NSF by the cell-free extract. This discrepancy might be explained if cerulenin were a competitive inhibitor of substrates since an apparently excess amount of acetoacetyl-CoA (0.3 mM) or acetyl-CoA (0.2 mM) was added in the enzyme assay mixture. However, when pre-incubation time of the enzyme with cerulenin was reduced from 30 min to 8 min, the inhibition was also reduced from 71% to 21%, and no pre-incubation resulted in no inhibition (Table 1). Therefore, inhibition appears to be non-competitive.

DISCUSSION. Although the discrepancy in cerulenin concentrations required for 50% inhibition of ^{14}C-acetyl-CoA incorporation into NSF and HMG-CoA synthase activity, the present study suggests that HMG-CoA synthase (condensing enzyme) appears to be the specific and only target of cerulenin

inhibition of overall sterol synthesis in yeast. Quite similar is the case of cerulenin inhibition of fatty acid synthesis in which β-ketoacyl-ACP synthetase (condensing enzyme) is the specific target of cerulenin in the fatty acid synthetase complex (6) and in the non-associated fatty acid synthetase (7). Cerulenin has been described to inhibit acyl-ACP:acyl-enzyme transacylation by binding to a site of β-ketoacyl-ACP synthetase of *E. coli* to which iodoacetamide and the acetyl moiety of acetyl-ACP could bind covalently (7). However, this is not likely in the case of cerulenin inhibition of HMG-CoA synthase activity since iodoacetamide inactivates acetoacetyl-CoA thiolase more strongly than HMG-CoA synthase present in the same preparation of these two enzymes (12), whereas cerulenin showed no inhibitory effect on the thiolase even at a concentration of 1.8 mM (Fig. 2). Both enzymes must have the component for acetyl-CoA:acetyl-enzyme transacetylation (13) to which iodoacetamide probably binds covalently. Moreover, the activity of acyl-CoA transacylase in the fatty acid synthetase complex of *M. phlei* was not affected by cerulenin when tested with model substrates (6). Therefore, cerulenin does not always seem to act by a common mechanism, i.e. inhibition of acyl-pantethein residue (CoA or ACP):acyl-enzyme transacylation, on condensing enzymes and it is conceivable that cerulenin does not inhibit acetyl-CoA:acetyl-enzyme transacetylation but rather inhibits only acetyl-enzyme:acetoacetyl-CoA condensation within the yeast HMG-CoA synthase.

ACKNOWLEGEMENT. The authors express their sincere thanks to Professor Toju Hata, Kitasato University, for his interest and continuous support throughout the work.

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