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THE ISOLATION OF TWO MUTANTS OF SACCHAROMYCES CEREVISIAE WHICH DEMONSTRATE INCREASED ACTIVITY OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE

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Summary. Two mutants of Saccharomyces cerevisiae have been isolated which demonstrate increased levels of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. The increased activity of this enzyme was paralleled by increases in sterol production. The mutants were selected by virtue of their resistance to the cytotoxic effects of 6-ketocholestanol. It was shown that this resistance results from decreased permeability of the cells to the oxygenated sterol analog. Gas-liquid chromatographic analyses of the sterols present in these strains implicate the conversion of squalene into sterols as an important regulatory step in sterol biosynthesis in yeast.

Introduction. The enzyme HMG-CoA reductase (3-hydroxy-3-methylglutaryl coenzyme A reductase, EC. 1. 1. 1. 34) has been the subject of intensive investigation in recent years as it is considered to be the key regulatory enzyme for the conversion of acetate to cholesterol (1). This enzyme catalyzes the formation of mevalonate from 3-hydroxy-3-methylglutaryl coenzyme A, and changes in the activity of HMG-CoA reductase determine overall rates of sterol synthesis (2,3). Certain oxygenated sterol derivatives such as 7-ketocholesterol and 25-hydroxycholesterol have been shown to be potent inhibitors of sterol synthesis in mammalian cells (4,5). In cell culture, such analogs are cytotoxic in the absence of various sterol supplements and have been shown to suppress the activity of HMG-CoA reductase (6).

Sinensky et. al. (7) and Cavenee et. al. (8) have isolated mutant cell lines resistant to the lethal effect of 25-hydroxycholesterol (7,8). These studies have suggested that the mutant strains are defective in some regulatory aspect of cholesterol synthesis involving HMG-CoA reductase. The activity of this enzyme in the mutant strains was elevated relative to the parent strains under conditions which normally depress sterol synthesis and enzyme activity (9,10).

Abbreviations. HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; NSF, non-saponifiable fraction

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In yeast, ergosterol synthesis has been extensively studied through the characterization of various mutant strains (11-14). Previous studies have implicated HMG-CoA reductase as the rate-limiting enzyme in this pathway (15,16). S. cerevisiae wild type strains are impermeable to exogenous sterols when grown aerobically. The erg mutant strains which cannot complete the terminal demethylation steps in ergosterol biosynthesis are also impermeable under aerobic conditions (14). Strain ole 3 was utilized in this study specifically due to its permeability properties under such growth conditions. This strain contains a lesion in porphyrin biosynthesis (17) and as a result cannot complete the terminal steps in ergosterol synthesis. The sterol pattern has been previously described and includes lanosterol and various methylated derivatives (18). Mutants of Candida have also been described which contained a similar pattern of methylated sterol intermediates (19). Using strain ole 3, mutants have been isolated which were resistant to the killing effect of 6-ketocholestanol.

Materials and Methods

Media: The medium used in these experiments contained 2% w/v Bacto-Peptone, 1% w/v Yeast Extract (Difco), 5% w/v Dextrose, plus 1% v/v Tween 80 (a polyoxyethylene derivative of oleic acid). All other chemicals and reagents were purchased from Sigma Chemical Company.

Yeast Strain: An isolate of strain ole 3 was utilized as the parent strain. This haploid strain is a porphyrin mutant which contains a lesion at δ-amino levulinic acid synthetase and has been previously characterized (18). As a result of this mutation, ole 3 cannot synthesize ergosterol which results in a membrane that is permeable to sterols under aerobic growth conditions.

Mutagenesis: Ethyl methane sulfonate (50% kill) was used to produce the mutant strains. Mutagenised cells were plated onto medium which contained 6-ketocholestanol (0.6mM). After approximately 3-5 days incubation at 30°C, colonies appeared. Two individual colonies were isolated and designated R6K18 and R6K6, respectively.

Growth Conditions: Cells were inoculated at an initial concentration of approximately 1×10^5 cells/ml and grown with shaking at 30°C to late log phase. Density determinations were made using a Klett-Summerson Photoelectric Colorimeter with a red filter. All readings were made over the range of 30-100 Klett units, which is known to be linear with cell concentration (20). Cells were harvested by centrifugation at 10,000 x g for 10 minutes at 5 °C and frozen until required.

Enzyme Isolation and Assay: Cells were thawed to room temperature and suspended to a density of 0.8 gram/ml in 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM dithiothreitol. A cell equivalent weight of pre-cooled glass beads (0.4 -0.5 mm) was added and the cells were disrupted in a Sorvall Omnimixer (setting 8) at 0° C for 10 minutes. The cell lysate was then incubated in 0.5% Triton X-100 for 60 minutes at 0° C. The fraction containing solubilized reductase was isolated and assayed as described by Quain and Haslam (21). A Pye Unicam Model SP1750 spectrophotometer was used with a Haake constant temperature circulator (30° C) to monitor the oxidation of NADPH at 340 nm.

Determination of Protein Concentration: Protein concentration was determined by the method of Lowry et. al. (22) following overnight dialysis against a change of distilled water at 4 °C to remove dithiothreitol. Bovine albumin was utilized as a standard.

Sterol Analysis: The procedures for extracting the non-saponifiable fraction (NSF) and quantifying the sterols present have been previously described (23). Gas-liquid chromatographic (GLC) analyses were performed on a Hewlett-Packard 5710A gas chromatograph. Non-derivatized samples of the NSF were dried under nitrogen, taken up in heptane, and chromatographed on a glass column (6 ft. x 4mm) of SE-30, 1% on gas-chrom Q (80/100 mesh) at 240 °C. The carrier gas was nitrogen with a flow rate of 60 ml/min. Retention times were compared to authentic standards supplied by Dr. Harold Pierce, Jr., Simon Fraser University. Sterols were quantified by weight determination of peak areas from original chromatograms.

Results and Discussion. Strain ole 3 was used to produce the mutant strains specifically because this strain is permeable to exogenous sterols when grown under aerobic conditions. Under anaerobic conditions wild type yeast strains are permeable to exogenous sterols but show only low levels of sterol synthesis. Strain ole 3 is sensitive to the killing effects of 6-ketocholestanol and 7-ketocholesterol. Following exposure to EMS, cells were plated onto medium containing 6-ketocholestanol (0.6mM). A number of resistant isolates were obtained, two of which, R6K6 and R6K18, were analyzed further. These two 6-ketocholestanol resistant strains were also resistant to 7-ketocholesterol.

As resistance to oxygenated derivatives of cholesterol in two mammalian cell lines has been correlated with changes in the activity of HMG-CoA reductase (9, 10), the specific activity of this enzyme was determined for the parent and mutant strains. The supernatant fraction (S_{8000}) described by Quain and Haslam (21) was shown to contain 84% of the total HMG-CoA reductase activity in solubilized yeast cells. The activities of this fraction for the three strains used are listed in Table I. Strain R6K6 showed a 2.7 fold increase in reductase activity relative to strain ole 3. Similarly, strain R6K18 demonstrated a 1.5 fold increase in enzyme activity.

Table I. Specific Activity of HMG-CoA reductase

Strain	Reductase Activity*	Fold Increase Relative to Strain ole 3
ole 3	8.6 ± 0.59 (5)	1.0
R6K18	13.1 ± 2.50 (3)	1.5
R6K6	23.3 ± 2.39 (5)	2.7

*Numbers represent n moles NADPH oxidized/min/mg protein $\pm$ standard deviation; numbers in parentheses represent the number of separate experiments.

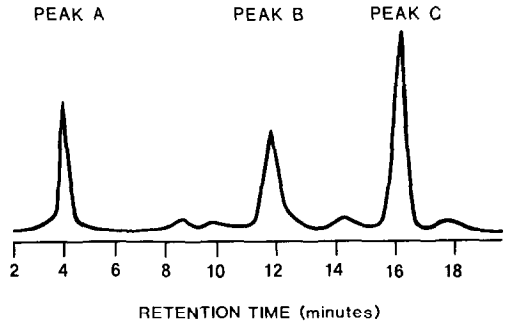

Figure I. Gas-Liquid Chromatogram of Free Sterols from Strain ole 3

Gas-liquid chromatographic analyses of the sterols present in these strains showed increases in NSF products corresponding to the observed alterations in HMG-CoA reductase activity. Figure 1 represents a typical chromatogram of the free sterols present in the NSF isolated from strain ole 3. R6K6 and R6K18 show similar chromatograms. Peak A corresponds to the retention time of squalene, peak B corresponds to the retention time of 4,14-dimethylzymosterol and 14-methylfecosterol, and peak C corresponds to the retention time of lanosterol. Peaks B and C contained 85% of the total sterols present in each strain. Four minor peaks comprised the remaining 15% of the total sterol present in the NSF. Table II represents a comparison of the sterol intermediates found in the three strains. The most significant change observed in the mutant strains was the several fold accumulation of squalene.

The inability of strain ole 3 to convert lanosterol to ergosterol results in the strain remaining permeable to exogenous sterols under aerobic conditions. Sterol analysis of the

Table II. Analysis of the Major Sterol Intermediates of Strains ole 3, R6K18, and R6K6

	Ratio of Major Sterol Products Relative to Strain ole 3		
Strain	squalene	4,14-dimethylzymosterol and 14-methylfecosterol	lanosterol
ole 3	1.0	1.0	1.0
R6K18	1.2	1.3	1.2
R6K6	7.7	2.8	1.1

sterol analyses performed as described in Materials and Methods.

resistant mutant strains grown in the presence of 6-ketocholestanol or ergosterol indicated that R6K6 and R6K18 no longer incorporated exogenous sterols. The observed increases in sterol production for the mutant strains apparently were paralleled by decreases in membrane permeability. This decreased permeability can account for the resistant phenotype.

In various studies, sites distal to HMG-CoA reductase have been implicated as additional regulatory sites in sterol biosynthesis. Specifically, lanosterol and zymosterol appear to be key intermediates in sterol synthesis in both yeast and various mammalian cell cultures (24-26). As the data suggests for the three strains observed, increases in HMG-CoA reductase activity are associated with increases in the amount of sterol accumulated. The large increase in squalene indicates that the conversion of squalene into the sterol fraction may be a highly regulated step. We have also observed the accumulation of squalene in other 6-ketocholestanol resistant mutants and in strain ole 3 grown in the presence of ergosterol. Further investigations are currently underway to elucidate the genetic and biochemical mechanisms involved in the regulation of HMG-CoA reductase activity.

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