Effects of Overproduction of the Catalytic Domain of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase on Squalene Synthesis in Saccharomyces cerevisiae

K. ALLEN G. DONALD,1,2* RANDOLPH Y. HAMPTON,2 AND IRVING B. FRITZ1

Department of Cellular Physiology, Babraham Institute, Babraham, Cambridge CB2 4AT, United Kingdom,1 and Department of Biology, University of California, San Diego, La Jolla, California 920372

Received 25 February 1997/Accepted 9 June 1997

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMG-R) is the major rate-limiting enzyme of the mevalonate pathway in many organisms, including yeasts. In the yeast Saccharomyces cerevisiae, there are two isoenzymes of HMG-R (Hmg1p and Hmg2p). Both consist of an anchoring transmembrane domain and a catalytic domain. We have removed the known controlling features of HMG-R by overproducing the catalytic domain of Hmg1p. This overproduction leads to an enhancement of squalene production, implying that HMG-R has been deregulated. The enhancement is apparent under semiaerobic and aerobic conditions. Despite the increase in squalene production, the amount of ergosterol produced by the HMG-R-overproducing yeast was not increased. This result suggests the presence of another regulatory step between squalene and ergosterol formation. Squalene levels generated by cells overproducing the catalytic domain of HMG-R were estimated to be up to 10 times those produced by wild-type cells. The enhancement in squalene production coincided with a reduction in growth rate. This reduction may be a direct consequence of the buildup of high concentrations of squalene and presqualene intermediates of the pathway.

The mevalonate pathway is responsible for the production of a huge array of substances in numerous organisms. In Saccharomyces cerevisiae, the pathway is responsible for the formation of many compounds, including ubiquinone, dolichol, and ergosterol (1). Squalene (an intermediate of ergosterol synthesis) and its derivative squalane are valuable compounds used in the cosmetic, pharmaceutical, and lubricant industries. Squalene rarely accumulates in nature, the notable exception being the cosmetic, pharmaceutical, and lubricant industries. In the presence of oxygen, under aerobic conditions, yeast is incapable of synthesizing squalene (1). An accumulation of squalene, under aerobic conditions, might be achieved by increasing the rate of flow through the early part of the mevalonate pathway, which might itself be accomplished by increasing the specific activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMG-R). Aerobically growing yeast cultures with enhanced HMG-R activity may produce industrially significant amounts of squalene, and examination of the biochemistries of these yeasts may further our knowledge of the regulation of the mevalonate pathway.

HMG-R is the key controlling enzyme in the mevalonate pathway (reviewed in reference 7). Two isoforms of HMG-R, Hmg1p and Hmg2p (encoded by the genes HMG1 and HMG2), exist in S. cerevisiae (1). Activity is regulated by numerous mechanisms: these include regulation of transcription (18), regulation of translation (5), and degradation of HMG-R in the endoplasmic reticulum (8). We hypothesized that by removing the DNA sequences necessary for these regulatory mechanisms, it may be possible to increase HMG-R activity and hence increase the rate of flow through the pathway. To test this hypothesis, we overexpressed the DNA sequence encoding the catalytic domain of Hmg1p (henceforth known as HMG1-CAT) in S. cerevisiae. We used the incorporation of [1-14C]acetate into lipids followed by thin-layer chromatography (TLC) to study the effect of this overexpression on squalene and ergosterol production.

MATERIALS AND METHODS

Materials. Squalene, ergosterol, HMG-CoA, NADPH, phenol-chloroform-isooctyl alcohol, ampicillin, and glass beads (diameter, 0.5 mm) were purchased from Sigma-Aldrich Co. Ltd. (Poole, Dorset, United Kingdom). 1-14C-nom acetate (specific activity, 59 mCi/mmol) and [a-32P]dCTP (specific activity, >3,000 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, Calif.). TLC plates (silica gel, 60 F254) were obtained from Merck (Darmstadt, Germany). A Ready-to-go DNA labeling kit was supplied by Pharmacia (Uppsala, Sweden).

Plasmid construction. Plasmid pRH127-3 contains a truncated HMG1 coding region (HMG1-CAT) encoding a soluble version of Hmg1p that includes only the linker and catalytic domain (Hmg1-35Glu). The truncated coding region has an amber codon. A truncated coding region was prepared from pRH102-1, which has the HMG1 coding region cloned into Bluescript II KS (Stratagene). The truncated coding region was then cloned into the pBluescript II KS (+) vector to create plasmid pRH127-3. Finally, pRH127-3 was prepared by cloning a 2.4-kb BamHI/SalI fragment of pRH127 into the BamHI and SalI sites of pBR322. The 2.4-kb BamHI/SalI fragment of pRH127-3 was prepared by annealing two synthetic oligonucleotides: 5’-GATCCATGGGCGTCA-3’ and 5’-GCCGATG-3’. Finally, pRH127-3 was prepared by cloning a 2-kb BamHI/SalI fragment of pRH127 into the BamHI and SalI sites of pBR322.

MATERIALS AND METHODS

Materials. Squalene, ergosterol, HMG-CoA, NADPH, phenol-chloroform-isooctyl alcohol, ampicillin, and glass beads (diameter, 0.5 mm) were purchased from Sigma-Aldrich Co. Ltd. (Poole, Dorset, United Kingdom). 1-14C-nom acetate (specific activity, 59 mCi/mmol) and [a-32P]dCTP (specific activity, >3,000 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, Calif.). TLC plates (silica gel, 60 F254) were obtained from Merck (Darmstadt, Germany). A Ready-to-go DNA labeling kit was supplied by Pharmacia (Uppsala, Sweden).

Plasmid construction. Plasmid pRH127-3 contains a truncated HMG1 coding region (HMG1-CAT) encoding a soluble version of Hmg1p that includes only the linker and catalytic domain (Hmg1-35Glu). The truncated coding region was prepared from pRH102-1, which has the HMG1 coding region cloned into Bluescript II KS (+) vector to create plasmid pRH127-3. Finally, pRH127-3 was prepared by cloning a 2.4-kb BamHI/SalI fragment of pRH127 into the BamHI and SalI sites of pBR322. The 2.4-kb BamHI/SalI fragment of pRH127-3 was prepared by annealing two synthetic oligonucleotides: 5’-GATCCATGGGCGTCA-3’ and 5’-GCCGATG-3’. Finally, pRH127-3 was prepared by cloning a 2-kb BamHI/SalI fragment of pRH127 into the BamHI and SalI sites of pBR322.

Received 25 February 1997/Accepted 9 June 1997

* Corresponding author. Mailing address: Department of Cellular Physiology, Babraham Institute, Babraham, Cambridge CB2 4AT, United Kingdom. Phone: (01223) 832312. Fax: (01223) 837912. E-mail: allen.donald@bbsrc.ac.uk.
bands were removed by scraping, and disintigrations per minute were established of standards and by the positions of bands on autoradiograms. The appropriate chloroform. Ergosterol and squalene were located on the plate by the migration and autoradiography. Lipids were dried under nitrogen and then resuspended in

For aerobic growth, 1 ml of cells was placed in a 50-ml tube at 30°C

These manipulations placed HMG1-CAT next to the glyceraldehyde-3-phosphate dehydrogenase promoter in a high-copy-number shuttle vector to allow high-level expression.

The plasmid pEMBLyex4 (kindly supplied by Jim Murray, Biotechnology Institute, Cambridge, United Kingdom) is a yeast shuttle vector (14), used only as a control in this report.

Strains and media. Both yeast strains described here were generated from AY926 (MATα ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100 ssd1-d2 gal 1). Strain AY127 was generated by transforming AY926 with pPH1273. Strain AYEMB was produced by transforming AY926 with pEMBLyex4. AYEMB was used as a nonoverproducing control that could grow in the same medium as AY127.

Yeasts were transformed by the dimethyl sulfoxide-enhanced lithium acetate method of Carlson and Botstein (4). Total RNAs were analyzed by gel transfer hybridization analysis according to the Hybond-N Northern blotting protocol method of Thorsness et al. (18). Cells were washed twice in 50 mM Tris-HCl (pH 7.5), 5 μl of NADPH (10 mM), and 5 μl of HMG-CoA (10 mM), 10 μl of cell extract was added. The starting point for measurement was the addition of cell extract, and the change in absorbance was recorded over a period of 1 min. The protein content of the cell extract was estimated by the method of Bradford (3) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The effects of overexpressing HMG1-CAT in S. cerevisiae were studied in terms of mRNA levels, HMG-R activity, and incorporation of radiolabeled acetate into squalene and ergosterol.

Total mRNAs from both AYEMB and AY127 early-stationary-phase cultures were probed with a 500-bp HindIII fragment located in HMG1-CAT. This probe will hybridize to HMG1-CAT transcripts of approximately 1.5 kb and to HMG1 transcripts of approximately 3 kb. A strong mRNA signal was detected in the Hmg1pcat-overproducing strain, AY127, at approximately 1.5 kb, but no mRNA species were detected in the control strain, AYEMB (Fig. 2a). This result suggests that the level of HMG1-CAT mRNA in AY127 is significantly higher than the level of HMG1 mRNA in either AY127 or AYEMB.

HMG-R activity was assayed by testing the ability of crude cell extracts to oxidize NADPH in the presence of HMG-CoA. HMG-R catalyzes the reduction of the thioester group of HMG-CoA in an NADP-dependent, four-electron reduction, which yields mevalonate. Assaying HMG-R activity from cultures grown under aerobic and semianaerobic conditions revealed that the AY127 (Hmg1pcat overproducer) extract had approximately 20 times the activity of the AYEMB (control) extract (Fig. 2b; the figure shows the result of aerobic growth, but semianaerobic growth produced the same difference in activity). This result correlates well with the mRNA levels and demonstrates that the normally tight regulation of HMG-R activity has been overcome by overproduction of the catalytic domain of HMG-R.

TLC studies of radiolabeled, nonsonifiable lipids demonstrated that AY127 consistently produced more squalene than AYEMB, while producing similar amounts of ergosterol. This increase in squalene was observed under semianaerobic and aerobic conditions (Fig. 3). Low-oxygen tension might explain the accumulation of squalene under semianaerobic conditions, as oxygen is required for the epoxidation of squalene and the activation of squalene epoxidase (10). However, under aerobic conditions, where oxygen should not be limiting, squalene would not normally accumulate. This result suggests that in the Hmg1pcat overproducer, AY127, one or a number of enzymes of the mevalonate pathway are subject to feedback inhibition and that this regulation may cause the accumulation of squalene. Several studies have demonstrated that certain enzymes of the mevalonate pathway are sensitive to ergosterol concentrations in the cell. M’Baya et al. have shown that both squalene epoxidase and squalene synthetase activity are inhibited by the presence of excess sterols (13). Those authors hypoth-
esized that the regulation took place at the level of transcription, as neither enzyme was inhibited by ergosterol in vitro. Dimster-Denk and Rine demonstrated that acetoacyl-CoA thiolase, the first enzyme of the pathway, is regulated at the level of transcription (6). The signal for regulation was found to be a sterol. Ergosterol has recently been identified as the effector molecule in the regulation of transcription of one of the late enzymes of the mevalonate pathway, C-5 sterol desaturase (encoded by the ERG3 gene). Smith et al. showed that the absence of ergosterol increased the transcription level of ERG3 35-fold (17).

Our results, as well as those described above, indicate that the mevalonate pathway is very sensitive to changes in the level of ergosterol within a yeast cell. It seems likely that at a critical concentration of ergosterol, many of the post-branch-point enzymes of the pathway are repressed. With this in mind, we propose the following model for squalene accumulation in yeast cells that overproduce the catalytic domain of Hmg1p. The presence of high levels of HMG-R activity leads to increases in the rate of flow through the pathway. When ergosterol concentrations reach a certain level in the cell, squalene epoxidase activity is repressed and squalene accumulates. In the AY127 yeast, the level of ergosterol is consistently high due to the strong constitutive promoter that controls HMG1-CAT expression. When [1-14C]acetate is added to the cell culture, AY127 cells can convert it to squalene at a higher rate than AYEMB cells can. Under semianaerobic conditions, oxygen is limiting and squalene is accumulated in both types of cell (Fig. 3). The squalene accumulation is accentuated in AY127 cells because they have higher levels of HMG-R activity. AY127 cells also form a smaller amount of ergosterol than AYEMB, possibly as a result of the buildup of toxic levels of squalene and presqualene compounds that inhibit flux through the later steps of the mevalonate pathway (see below). Under aerobic conditions, new cell growth and a good supply of oxygen allow the synthesis of larger amounts of ergosterol (Fig. 3). Despite this increase in ergosterol formation, AY127 cells accumulate squalene to levels similar to those accumulated under semianaerobic conditions (Fig. 3). The similar levels of squalene formed under both aerobic and semianaerobic conditions may be due to ergosterol repressing feedback inhibition of squalene epoxidase. The buildup of squalene may be accompanied by the accumulation of precursors of squalene. This feedback regulation may be particularly true of farnesyldiphosphate accumulation, since the enzyme converting it into squalene (squalene synthetase) has also been shown to be sensitive to levels of ergosterol (13).

This model might also explain another phenomenon of the Hmg1pCate overproducer: AY127 consistently grows more slow-

FIG. 2. The effect of the overexpression of HMG1-CAT and the consequent overproduction of the catalytic domain of Hmg1p on mRNA and HMG-R activity. Crude cell extract and mRNA were isolated from Hmg1pCate-overproducing (AY127) and wild-type (AYEMB) yeasts. Two micrograms of total mRNA was loaded into an agarose-formaldehyde gel for Northern blot analysis. Following hybridization, HMG1 species were detected with a 500-bp DNA probe from the catalytic domain region (a). A hybridization control was carried out with the yeast ACT1 gene. The ACT1 transcript was present in equal amounts in total RNAs isolated from both strains (data not shown). HMG-R activity was estimated by the reduction in A340 accompanying the oxidation of NADPH (b). Error bars represent ± 1 standard deviation, which was calculated from four repeat experiments. The units (i) are for A340.

FIG. 3. Effects of overproduction of the catalytic domain of Hmg1p on squalene and ergosterol levels. Nonsaponifiable lipids were extracted from Hmg1pCate-overproducing (AY127) and wild-type (AYEMB) yeasts. The extraction was carried out after growth under conditions without shaking (semianaerobic) and with vigorous shaking (aerobic). Incorporation of [14C]acetate into nonsaponifiable lipids was detected by autoradiography of TLC plates, and the bands corresponding to squalene and ergosterol were removed for scintillation counting. The results are the averages of three independent experiments.
ly than AYEMB. One possible reason for this slower growth is that squalene, or one of the precursors of squalene (such as farnesylphosphate), is cytotoxic. Thus, when these compounds accumulate in large amounts in AY127, they lower the cell growth rate. We are currently investigating the effect of compounds that would allow industrially significant amounts of squalene to be produced.

Experiments examining unlabeled nonsaponifiable lipids revealed similar patterns of distribution of squalene and ergosterol in AY127 and AYEMB growing under highly aerobic conditions (results not shown). Equal amounts of cells produced equal amounts of ergosterol, but squalene levels were higher in AY127. We estimate levels of squalene in the dry cells. With a controllable promoter and limited aerobic conditions, it should be possible to develop a fermentation system that would allow industrially significant amounts of squalene to be produced.

ACKNOWLEDGMENTS

This work was supported by a ROPA grant (reference no. MOL04571) from the Biotechnology and Biological Science Research Council of the United Kingdom.

We thank Ruth Mould and Peter White for their constructive comments on the manuscript. Thanks also go to Mike Stark for providing the yeast strain (via Andy Carter).

REFERENCES