Regulation of Ergosterol Biosynthesis and Sterol Uptake in a Sterol-Auxotrophic Yeast†

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Inhibition of sterol uptake in Saccharomyces cerevisiae sterol auxotroph FY3 (α heml erg7 ura) by δ-aminolevulic acid (ALA) is dependent on the ability of the organism to synthesize heme from ALA. Sterol-depleted cells not exposed to ALA or strain PFY3 cells, with a double heme mutation, exposed to ALA did not exhibit inhibition of sterol uptake. Addition of ALA to sterol-depleted FY3 stimulated production of a high endogenous concentration of 2,3-oxidosqualene (25.55 μg mg−1 [dry weight]) at 24 h, whereas FY3 not exposed to ALA or PFY3 exposed to ALA did not accumulate 2,3-oxidosqualene. The high concentration of 2,3-oxidosqualene in FY3 with ALA decreased, and 2,3:22,23-dioxidosqualene increased to a very high level. The elevation of 2,3-oxidosqualene by ALA was correlated with a fivefold increase in the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (EC 1.1.1.34). The enhanced activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase was prevented by cycloheximide but not chloramphenicol and was dependent on a fermentative energy source. Inhibition of sterol uptake could not be attributed to 2,3-oxidosqualene or 2,3:22,23-dioxidosqualene but was due to a nonsaturating level of ergosterol produced as a consequence of heme competency through a leaky erg7 mutation.

Biosynthesis of sterols and unsaturated fatty acids is an aerobic process in Saccharomyces cerevisiae (1). Hemoproteins are known to participate in several steps of the sterol biosynthetic pathway, including dehydrogenation and demethylation reactions (13, 21). Thus, sterol biosynthesis is precluded in anaerobically cultured wild-type cells or in heme-deficient mutants cultured aerobically because of lack of the corresponding hemoproteins (2, 3, 12). Previous studies have indicated that when wild-type cells are grown aerobically, they are apparently impermeable to exogenous sterols (17, 27). Heme mutants, however, accumulate exogenous sterols from the medium under aerobic conditions (9, 26).

We have shown recently that heme deficiency allowed sterol uptake in a sterol auxotroph (15) and that cholesterol uptake was inversely proportional to the initial endogenous cellular concentration of cholesterol in a sterol-auxotrophic strain (16). That is, conditions that result in sterol-depleted cells or an endogenous sterol concentration below the saturation level allowed exogenous sterol uptake. Wild-type yeasts are capable of maintaining a free sterol saturation level throughout the growth cycle by using the sterol ester pool as a reserve source of free sterol (24) and thus do not take up exogenous sterol. However, in the heme mutant FY3 (heml erg7), which is additionally defective in 2,3-oxidosqualene cyclase, it has been found that, when δ-aminolevulic acid (ALA) is supplied to allow heme synthesis, sterol uptake is inhibited (15).

To understand the mechanism by which heme inhibits sterol uptake, a process known to be regulated only by the endogenous sterol concentration, we examined the effect of allowing heme biosynthesis on the activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA; EC 1.1.1.34) reductase in this sterol-auxotrophic strain.

MATERIALS AND METHODS

Yeast strains. S. cerevisiae FY3 (α heml erg7 ura met) was used in this study and has been described before (25). Strain PFY3 was derived from FY3 and has an additional heme mutation between the steps of ALA permeation and insertion of iron into protoporphyrin IX (15).

Media and growth conditions. The medium used for growth and sterol uptake assays consisted of 2% glucose, 0.67% yeast nitrogen base (Difco Laboratories), and 1% Casamino Acids (Difco) and was supplemented with 20 μg each of uracil and methionine per ml. The medium was buffered with 0.05 M succinic acid and adjusted to pH 5.5. Cholesterol and ergosterol were recrystallized and added to the growth medium at the designated concentration in Tergitol-4-Nondet P-40-95% ethanol (1:1 [vol/vol]). Unsaturated fatty acid supplementation consisted of a mixture of oleic and palmitoleic acids (4:1 [vol/vol]) at a 0.01% final concentration added from a 10% (vol/vol) solution in Tergitol 4-ethanol (1:1 [vol/vol]). ALA was dissolved in distilled water and filter sterilized (0.45-μm [pore size] Millipore HA) before addition to the media.

Cultures were grown aerobically with rotary shaking at 28°C, and growth was monitored with a Klett-Summerson photoelectric colorimeter equipped with a green filter. Klett units were found to be proportional to cell number by the following formula: log cells per ml (log Klett units + 1.86)/0.6486 over a range of 5 to 500 Klett units, as verified by direct cell counts with a Coulter counter.

Assay of sterol uptake. Cells to be assayed for sterol uptake were grown on the appropriate concentration of cholesterol or ergosterol and exposed to cycloheximide (200 μg ml−1) in late-exponential phase for 2 h to arrest growth. Cells were then harvested by centrifugation and washed twice with a 1% aqueous solution of Tergitol-ethanol (1:1 [vol/vol]). Cells were suspended in sidearm flasks containing fresh medium and cycloheximide (200 μg ml−1). The assay was started by addition of a mixture of [4,14C]cholesterol (0.1 μCi ml−1) and unlabeled cholesterol (15 μg ml−1). Cells were typically

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added to a density of 140 Klett units. Assay cultures were incubated at 28°C on a rotary shaker, and 1.0-ml samples were taken periodically, filtered (0.45-μm Millipore HA), and washed with 50 ml of a 1% aqueous solution of Tergitol 4-ethanol (1:1 [vol/vol]). Filters were collected and dried in scintillation vials before counting in a Packard Tri-Carb 300 liquid scintillation spectrometer with toluene-PPO (2,5-diphenyloxazole)-POPOP [1,4-bis(5-phenyloxazolyl)benzene]. The amount of sterol taken up per cells was measured as (counts per minute – background counts per minute)/number of cells.

Quantification of lipids. Cells used for quantification of lipid content were harvested by centrifugation and washed twice with a 1% aqueous solution of Tergitol-ethanol (1:1 [vol/vol]). The washed cell pellets were frozen, lyophilized, and steam dried in the presence of dimethyl sulfoxide, followed by hexane extraction as previously described (18). Purified extracts were resolved by thin-layer chromatography on silica gel plates with cyclohexane-ethyl acetate (85:15 [vol/vol]) as the solvent system. Bands (visualized by UV light or exposure to iodine) were scraped and eluted with chloroform-methanol (4:1 [vol/vol]). The purified extracts were dried under nitrogen and dissolved in chloroform before injection into a Varian 2700 gas chromatograph coupled to an IBM CS 9000 chromatography work station for quantification. An SE-30 column was used with a carrier gas flow rate of 30 ml min⁻¹ and a column temperature of 235°C; the injector and detector were at 250°C. Standard calibration curves were used to quantify cellular concentrations of sterols or sterol precursors.

High-performance liquid chromatographic purifications. Purification of sterol precursors was done by high-performance liquid chromatography and has already been described (22).

Exposure to antibiotics. Cycloheximide was used at 300 μg ml⁻¹, and chloramphenicol was used at 400 μg ml⁻¹ in distilled water after filter sterilization (0.45-μm Millipore HA).

Chemicals. Cholesterol, ergosterol, Tergitol 4-Nonidet P-40, chloramphenicol, cycloheximide, ALA, and amino acids were purchased from Sigma Chemical Co. Solvents were obtained from Fisher Scientific Co. and redistilled before use. [4-¹⁴C]cholesterol was a product of New England Nuclear Corp. Silica gel plates were from E. Merck AG.

RESULTS

Effect of ALA on sterol uptake by sterol-depleted FY3. When sterol-depleted cells (16) were exposed to a range of ALA concentrations for 48 h, harvested, and then assayed for sterol uptake, a concentration of 100 μg of ALA per ml exhibited maximal inhibition of sterol uptake. ALA was used at 100 μg ml⁻¹ for the remainder of the study. FY3 was also pregrown on ergosterol (15 μg ml⁻¹) and assayed for sterol uptake. These cells were only slightly more inhibited for sterol uptake than the sterol-depleted culture exposed to ALA (100 μg ml⁻¹) only (Fig. 1). Strain PFY3, which has been reported to have an additional heme mutation between the steps of ALA permeation and iron insertion into protoporphyrin IX, was not inhibited for sterol uptake by ALA. Sterol-depleted FY3 not exposed to ALA was used as a control for sterol uptake determinations.

Lipid composition of ALA-inhibited FY3. To determine whether ALA-exposed cells produced a lipid component that prevented sterol uptake, FY3 cells fed ALA for 48 h were extracted with hexane (18), and the extract was added to the culture medium (in Tergitol 4-ethanol, 1:1 [vol/vol]) with sterol-depleted cells. A sterol uptake assay was performed on the extract-fed cells after exposure for 48 h. Cultures exposed to the extract were as inhibited for sterol uptake as were the cultures fed 100 μg of ALA per ml for 48 h (data not shown).

Gas chromatographic analysis of the lipid extract showed two unusual components that did not correspond to the elution time of ergosterol or cholesterol. Mass spectral analysis gave fragmentation patterns identical to those which have been reported for the sterol precursors 2,3-oxidosqualane and its derivative 2,3,22,23-dioxidosqualene (DOS) (data not shown; 10, 11). A third peak of much smaller area corresponded to ergosterol in elution time and mass fragmentation pattern.

To examine the appearance and concentration of the squalene epoxidation products, a large culture of sterol-depleted FY3 was exposed to ALA (100 μg ml⁻¹), and samples were taken periodically, extracted, and quantified by gas chromatography (Fig. 2). The cellular concentration of 2,3-oxidosqualene rose dramatically during this period, reaching a peak at about 24 h and declining thereafter. The cellular concentration of DOS remained low initially and then steadily increased to a plateau. The intracellular concentration of ergosterol rose linearly from 0.008 ± 0.002 to 1.29 ± 0.059 μg mg⁻¹ (dry weight) during this experiment. However, when sterol-depleted FY3 was exposed to ALA in the presence of 2.0% ethanol or glycerol instead of glucose, there was no accumulation of 2,3-oxidosqualene, DOS, or ergosterol. Sterol uptake assays performed on these cells revealed that they were not inhibited for cholesterol uptake.

Identification of the sterol uptake inhibitor. To determine which compound caused inhibition of sterol uptake, 2,3-oxidosqualene and DOS were purified by high-performance liquid chromatography and individually added to cell suspensions of sterol-depleted FY3 for 48 h. These cells were then assayed for sterol uptake. There were no significant differences in sterol uptake between control cells and cells exposed to 100 μg of purified 2,3-oxidosqualene or DOS per ml (data not shown).

Sterol-depleted FY3 cells were then exposed to a range of

![FIG. 1. Sterol uptake assay of strains used under the following conditions: sterol-depleted FY3 without ALA or double heme mutant PFY3 exposed to 100 μg of ALA per ml for 48 h (△), FY3 exposed to 20 μg of ALA per ml for 48 h (▲), FY3 exposed to 40 μg of ALA per ml for 48 h (●), FY3 exposed to 100 μg of ALA per ml for 48 h (○), and FY3 pregrown on 15 μg of ergosterol per ml (■).]
ergosterol concentrations, harvested after 48 h, and extracted to quantify their respective endogenous ergosterol concentrations (Fig. 3). The saturation level for ergosterol was about 2.5 μg mg⁻¹ (dry weight). Cells fed 5.0 μg of ergosterol per ml had an endogenous ergosterol concentration similar to that of cells exposed to 100 μg of ALA per ml for 48 h (1.18 μg mg⁻¹ [dry weight]), and therefore we tested cells fed different concentrations of ergosterol for their ability to take up cholesterol. We found that the nonsaturating concentration of ergosterol found in the free-sterol fraction of FY3 fed 5.0 μg of ergosterol per ml could fully account for the inhibition of cholesterol uptake observed when FY3 was exposed to 100 μg of ALA per ml for 48 h (data not shown).

Evidence for a heme-regulated step early in the ergosterol biosynthetic pathway. Since elevation of cellular 2,3-oxidosqualene was dependent on heme competency, and since HMG-CoA reductase is known to be a highly regulated enzyme (14, 19, 23), HMG-CoA reductase activity was assayed (20). The specific activity of HMG-CoA reductase in ALA-exposed cells increased approximately fivefold relative to that of heme-deficient control cells (Table 1). Stimulation of HMG-CoA reductase activity required glucose as the energy source; 2% glycerol or ethanol did not allow increased activity. Cycloheximide, but not chloramphenicol, prevented this heme-stimulated increase in activity. The specific activity of HMG-CoA reductase in strain PFY3, which cannot attain heme competency with ALA, was not stimulated.

Sterol-depleted FY3 pretreated with 300 μg of cycloheximide per ml for 4 h before exposure to ALA did not accumulate 2,3-oxidosqualene or DOS or produce ergosterol; an assay performed on these cells revealed that they were not inhibited for sterol uptake. However, when sterol-depleted FY3 was pretreated with 400 μg of chloramphenicol per ml for 4 h before exposure to ALA, accumulation of 2,3-oxidosqualene did proceed, followed by conversion to DOS and production of about 1.2 μg (dry weight) of ergosterol per mg. A sterol uptake assay performed on these cells showed that they were as inhibited as control cells not pretreated with chloramphenicol before exposure to ALA (data not shown).

DISCUSSION

HMG-CoA reductase has been implicated as a primary regulatory site in the sterol biosynthetic pathway (23). Ergosterol has been reported to function as a feedback inhibitor (14, 19), with glucose or unsaturated fatty acids acting as inducers of activity (4–6). Other researchers have reported that squalene epoxidase may be another highly regulated enzyme because of the requirement for unsaturated fatty acids in the conversion of squalene to 2,3-oxidosqualene, although the exact mechanism is not known (7).

Analysis of FY3 under sterol-depleted conditions (16) in the presence of glucose and unsaturated fatty acids showed that sterol precursors did not accumulate to a high level unless ALA was added to the medium to alleviate the heme mutation and that 2,3-oxidosqualene was the main precursor initially produced. Squalene did not accumulate with or without ALA supplementation. Additionally, glucose was found to be necessary for stimulation of HMG-CoA reductase and accumulation of elevated levels of 2,3-oxidosqualene. When ethanol or glycerol was the energy source, there was no stimulation of the enzyme with ALA present (Table 1). Others have reported that glucose itself is the inducer of HMG-CoA reductase (4).

To determine whether ALA itself or heme was the inducer of HMG-CoA reductase, an assay was performed on FY3 and PFY3 grown in media containing various carbon sources and exogenous ALA (Table 1). The specific activity of ALA (100) was 0.12±0.01, while that of PFY3 was 0.08±0.01. The procedure for the HMG-CoA reductase assay was from Quain and Haslam (20) and was carried out after 16 h under the designated conditions.

![Image](https://example.com/image1.png)

FIG. 2. Quantification of sterol intermediates extracted from sterol-depleted strains exposed to 100 μg of ALA per ml. Intermediates: 2,3-oxidosqualene from FY3 (●), DOS from FY3 (●), and total 2,3-oxidosqualene and DOS from PFY3 (△).

![Image](https://example.com/image2.png)

FIG. 3. Intracellular ergosterol extracted from FY3 grown on different concentrations of ergosterol and harvested at late-exponential phase.

| Supplement(s) (concn [μg ml⁻¹]) | Carbon source (2%) | Strain | Sp act (nmol/min per mg of protein) |
|---------------------------------|-------------------|--------|-----------------------------------|
| None                            | Glucose FY3       | 1.04 ± 0.09 |
| ALA                             | Glucose FY3       | 5.28 ± 0.37 |
| ALA (100) + CH (300)            | Glucose FY3       | 0.96 ± 0.08 |
| ALA (100) + CP (400)            | Glucose FY3       | 5.08 ± 0.32 |
| None                            | Glucose PFY3      | 0.78 ± 0.13 |
| ALA (100)                       | Glucose PFY3      | 0.88 ± 0.14 |
| None                            | Ethanol FY3       | 0.74 ± 0.11 |
| ALA (100)                       | Ethanol FY3       | 0.96 ± 0.12 |
| None                            | Glycerol FY3      | 0.72 ± 0.11 |
| ALA (100)                       | Glycerol FY3      | 0.98 ± 0.14 |

* Cells were pretreated with cycloheximide (CH) or chloramphenicol (CP) for 4 h, as indicated, before exposure to ALA.
* The procedure for the HMG-CoA reductase assay was from Quain and Haslam (20) and was carried out after 16 h under the designated conditions.
of HMG-CoA reductase, the double heme mutant PFY3 was exposed to ALA and analyzed for enzyme activity and production of 2,3-oxidosqualene (Table 1; Fig. 2). There were no significant differences between ALA-exposed PFY3 and the control not exposed to ALA, indicating that the inducer was not ALA but heme or a heme product such as cytochromes. Thus, if accumulation of the sterol intermediate 2,3-oxidosqualene is used as a measure of the flow of metabolites through the ergosterol pathway, then hemes or heme products are necessary not only in several steps in the ergosterol pathway (13, 21) but also as a positive regulator of ergosterol biosynthesis.

It was also shown that pretreatment with cycloheximide before exposure to ALA inhibited production of 2,3-oxidosqualene and, subsequently, production of DOS and ergosterol, and sterol uptake was not inhibited. Cycloheximide is known to inhibit cytoplasmic protein synthesis specifically (8). However, pretreatment with chloramphenicol (which selectively inhibits mitochondrial protein synthesis in S. cerevisiae) before ALA exposure did not inhibit production of 2,3-oxidosqualene, DOS, or ergosterol. An assay performed on these cells revealed that they were inhibited for sterol uptake. These results confirm those of other researchers who reported that induction of HMG-CoA reductase requires cytoplasmic protein synthesis when anaerobically grown cells are shifted to aerobic conditions (5, 6).

The high levels of 2,3-oxidosqualene allowed synthesis of DOS, which is usually not found in cells unless very high levels of 2,3-oxidosqualene are produced. DOS is thought to be produced by the squalene epoxidase enzyme (10, 11). The greatly elevated concentration of 2,3-oxidosqualene also allowed ergosterol to be produced through the leaky erg7 mutation (up to 1.29 µg/ml [dry weight]), which inhibited uptake of exogenous cholesterol. Regulation of cholesterol uptake is known to be inversely proportional to the concentration of intracellular free sterol (16). FY3 without ALA or PFY3 with ALA did not accumulate a high background of 2,3-oxidosqualene and thus could not synthesize ergosterol.

The nonsaturating amount of ergosterol synthesized when FY3 is exposed to ALA could fully account for sterol uptake inhibition when heme competency is acquired. Feeding high concentrations of high-performance liquid chromatography-purified 2,3-oxidosqualene or DOS to sterol-depleted FY3 did not inhibit sterol uptake.

We were able to demonstrate that, although glucose is required for induction of HMG-CoA reductase, heme or a heme product, such as cytochromes, was responsible for the fivefold increase in activity. Our results confirm that heme competency is required for sterol uptake inhibition and that inhibition is directly caused by the endogenous ergosterol produced. We are currently investigating the cause of ALA-induced growth inhibition in FY3 (15), which may be related to accumulation of 2,3-oxidosqualene within the first 24 h of incubation with ALA.

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