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Yeast Oxidosqualene Cyclase (Erg7p) Is a Major Component of Lipid Particles*

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Oxidosqualene cyclase of the yeast encoded by the *ERG7* gene converts oxidosqualene to lanosterol, the first cyclic component of sterol biosynthesis. In a previous study (Athenstaedt, K., Zweytick, D., Jandrositz, A, Kohlwein, S. D., and Daum, G. (1999) *J. Bacteriol.* 181, 6441–6448), Erg7p was identified as a component of yeast lipid particles. Here, we present evidence that Erg7p is almost exclusively associated with this compartment as shown by analysis of enzymatic activity, Western blot analysis, and *in vivo* localization of Erg7p-GFP. Occurrence of oxidosqualene cyclase in other organelles including the endoplasmic reticulum is negligible. In an *erg7* deletion strain or in wild-type cells treated with an inhibitor of oxidosqualene cyclase, the substrate of Erg7p, oxidosqualene, accumulated mostly in lipid particles. Storage in lipid particles of this intermediate produced in excess may provide a possibility to exclude this membrane-perturbing component from other organelles. Thus, our data provide evidence that lipid particles are not only a depot for neutral lipids, but also participate in coordinate sterol metabolism and trafficking and serve as a storage site for compounds that may negatively affect membrane integrity.

Oxidosqualene cyclases (OSCs)[1] play a central role in sterol biosynthesis because they catalyze a key reaction of the sterol biosynthetic pathway, namely formation of a cyclic component from the acyclic precursors oxidosqualene and dioxidosqualene (Fig. 1). OSCs may be regarded as phylogenetic markers insofar as they form a variety of cyclic triterpenoids in plants, whereas their only cyclization product in nonphotosynthetic eukaryotes such as animals, yeast, and other fungi is lanosterol (1). During the last 20 years studies on OSCs focused on several biochemical, molecular biological, and cell biological aspects. Hundreds of inhibitors were synthesized and tested for their effect on this enzyme (2–6), OSCs from different sources were characterized and purified (7–10), and genes encoding these proteins in various species were cloned and sequenced (11–15). During these studies, however, the important question as to the subcellular localization of this enzyme was surprisingly ignored. This aspect is essential because localization of enzymes involved in the sterol biosynthetic pathway may contribute greatly to the understanding of coordination and regulation of this pathway and of sterol traffic among organelles (16–19).

Subcellular localization of yeast OSC, which is encoded by the *ERG7* gene, stimulated our interest not only because Erg7p is one of the most important enzymes of sterol biosynthesis that has not been localized so far, but also because targeting of the protein to its proper cellular environment may help to explain its mode of action and to design new inhibitors of OSC. As an example, we reported recently that some OSC inhibitors that undergo metabolic transformations in the yeast exhibit different effects because of different subcellular distribution of target proteins and metabolizing enzymes, respectively (20).

The question as to the subcellular localization of yeast OSC was first addressed more than 30 years ago by Shechter *et al.* (21). These authors compared properties of yeast and mammalian enzymes and concluded that the yeast enzyme was soluble, whereas the mammalian enzyme was bound to microsomes. Properties of the apparently soluble yeast OSC, however, turned out to be quite different from "true" soluble cytosolic enzymes. These results suggested that yeast OSC may be a particle-bound enzyme that becomes soluble during cell disruption and fractionation of the homogenate. Experiments in our laboratory (22) revealed that the occurrence of the two forms (soluble and membrane-bound) of OSC in yeast, which can be distinguished by their sedimentation properties, largely depends on the protocol used for the preparation of the microsomal fraction. A clue to the understanding of yeast OSC localization came from the biochemical characterization of the so-called lipid particles, a subcellular compartment highly enriched in triacylglycerols and steryl esters which harbors several enzymes of lipid metabolism (17, 19, 23). Among these proteins, two enzymes of ergosterol biosynthesis, namely sterol Δ^{24}-methyltransferase (Erg6p) and squalene epoxidase (Erg1p), were identified as typical lipid particle proteins. It has to be mentioned, however, that Erg6p is almost exclusively localized to this compartment, whereas Erg1p partitioned between lipid particles and the endoplasmic reticulum (24). Recently, the yeast OSC, Erg7p, was also identified as a lipid particle protein by a mass spectrometry approach (23), but the distribution of the protein between lipid particles and the endoplasmic reticulum or other organelles was not determined.

In the present study, we provide biochemical and cell biolog-

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[1] The abbreviations used are: OSC(s), oxidosqualene cyclase(s); GFP, green fluorescent protein; PCR, polymerase chain reaction; MES, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography.

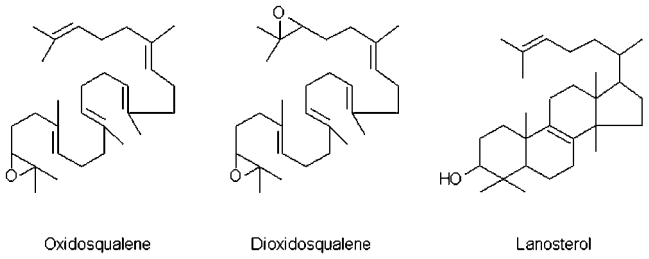

FIG. 1. **Intermediates of sterol metabolism at the stage of conversion of acyclic to cyclic components.**

ical evidence supporting the localization of OSC in yeast lipid particles. We demonstrate by enzyme measurements and immunological methods that only marginal amounts of OSC are present in the endoplasmic reticulum. The consequence of an *erg7* deletion and the inhibition of OSC by the inhibitor U14266A (3β-(2-dimethylaminoethoxy)-androst-5-en-17-one) (25) on the subcellular deposition of the precursors oxidosqualene and dioxidosqualene as well as the effect of anaerobiosis on the subcellular distribution of Erg7p are discussed.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—The wild-type strain *Saccharomyces cerevisiae* FL100 (*MATa ura3–1 trp1–1*) and the *erg7* mutant (*MATa erg7 ura3–1 trp1–1*) were kindly provided by F. Karst, Colmar, France (26). Cells were grown aerobically or anaerobically to the early stationary phase at 30 °C in YPD medium containing 1% yeast extract (Oxoid, England), 2% peptone (Oxoid), 2% glucose (Merck, Darmstadt, Germany), and 20 μg/ml ergosterol added from a stock solution of 2 mg/ml ergosterol in ethanol:Tween 80 (1:1; v/v). Inocula (0.5 ml/100 ml of culture medium) were made from precultures grown aerobically or anaerobically for 18 or 22 h, respectively.

Construction of a Yeast Strain Containing Erg7p-GFP—For the construction of Erg7p-GFP, the GFP(S65T) sequence and the selectable marker kanMX6 were inserted into the chromosomal DNA of the coding region of *ERG7* at the N terminus and the C terminus, respectively. The fragment for insertion was amplified by PCR with the plasmid pFA6a-kanMX6-PGAL1-GFP(S65T) for N-terminal, or with the plasmid pFA6a-GFP(S65T)-kanMX6 for C-terminal tagging (27). Primers used for amplification are shown in Fig. 2. PCR was performed as described previously (23). PCR fragments were ethanol precipitated, and 400–700 ng was used for transformation of FY1679 applying the high efficiency lithium acetate transformation protocol (28). Transformed cells were grown in YPD at 30 °C overnight and then spread on YPD plates containing 200 mg of G418 (Invitrogen) per liter. After incubation for 2–3 days, large colonies were transferred to fresh YPD-G418 plates. Clones that yielded colonies were considered as positive transformants and checked further for correct integration of the respective fusion cassette. Correct replacement was verified by analytical PCR of whole yeast cell extracts (29).

Microscopy—ERG7-GFP-expressing cells were harvested after 16 h (YPD), immobilized under agarose, and stained with the lipophilic dye Nile Red as described (30), and analyzed by confocal laser scanning microscopy using a Leica TCS4d confocal microscope. GFP fluorescence was monitored at 488 nm excitation by using a 525/50 band pass filter. Nile Red fluorescence was excited by the 547 nm line of the Ar/Kr laser, attenuated to 5% of total output power, and detected using a 590 nm long pass filter. Transmission images were recorded using Nomarski (differential interference contrast) optics. Because of the weak GFP signal, cells were scanned sequentially for GFP and Nile Red fluorescence. Images were edited using Adobe Photoshop.

Isolation of Yeast Subcellular Fractions—Lipid particles and vacuoles were obtained at high purity by the method of Leber *et al.* (19). In brief, yeast cells were grown in rich medium (YPD) to the late exponential phase and converted to spheroplasts (31). Spheroplasts were washed twice with 20 mM potassium phosphate (pH 7.4) and 1.2 M sorbitol and then homogenized in breaking buffer containing 10 mM MES/Tris (pH 6.9), 12% Ficoll 400, 0.2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride at a final concentration of 0.5 g of cell wet weight/ml. The homogenate was centrifuged at 5,000 × *g* for 5 min, and the resulting supernatant was overlaid with breaking buffer and centrifuged for 1 h at 28,000 rpm (100,000 × *g*) in an SW-28 swing-out rotor. The floating layer containing lipid particles and vacuoles was collected from the top of the gradient and suspended in breaking buffer. Lipid particles were separated from vacuoles by two additional sequential flotation steps in (i) 10 mM MES/Tris (pH 6.9), 8% Ficoll 400, 0.2 mM EDTA, and (ii) 10 mM MES/Tris (pH 6.9), 0.25 M sorbitol, 0.2 mM EDTA at 28,000 × *g* for 30 min, each. The floating layer from the top of the last gradient contains lipid particles at high purity; vacuoles are obtained in the pellet at all three flotation steps.

		Primer (5' ---> 3')
Erg7p–GFP (N-term)	fw	GTACTGCTGTGCCCAATAACCTTACCAATAATC GTCGCCCACAAAGAAAGTACAAAACAGGAATT CGAGCTCGTTTAAAC
	rev	CAGTCTCCAAAGACGTGGATCTGTCTTTGGTA GACCGATTGTGTCAGAATAAAATTCTGTTTTGT ATAGTTCATCCATGC
Erg7p–GFP (C-term)	fw	CGATTCTTATTCCCTATTAAGGCATTAGGTATG TACAGCAGGGCATATGAAACACATACGCTTCG GATCCCCGGGTTAATTAA
	rev	ATACTAGTTTTGCACTAGTTTCTAATTGTTGCA GCCTCTAACAACACTTATAAATAAAACGAATT CGAGCTCGTTTAAAC

FIG. 2. **Primers used for the construction of *ERG7-GFP*.** *fw*, forward direction; *rev*, reverse direction.

For the preparation of cytosol and microsomal fractions, yeast cells were grown in YPD and converted to spheroplasts as described by Daum *et al.* (31). Spheroplasts were homogenized (19), and the homogenate was centrifuged for 30 min at 20,000 × *g*. The resulting supernatant was subjected to sequential centrifugations at 30,000 × *g* for 30 min, 40,000 × *g* for 30 min, and 100,000 × *g* for 45 min. The resulting pellets were used as 30,000 × *g*, 40,000 × *g*, and 100,000 × *g* microsomes, respectively. To obtain highly purified 30,000 × *g* microsomes the respective pellet was subjected to two additional washing steps by suspending in 10 mM Tris/HCl (pH 7.5) and centrifugation at 30,000 × *g*. The particle-free 100,000 × *g* supernatant is the cytosolic fraction.

Characterization of Subcellular Fractions—Protein was quantitated by the method of Lowry *et al.* (32) using bovine serum albumin as a standard. Proteins of microsomal and cytosolic fractions were precipitated from the aqueous phase with trichloroacetic acid (final concentration 10%) and solubilized in 0.1% SDS and 0.1 M NaOH. Prior to protein analysis of the lipid particle fraction, samples were delipidated. Nonpolar lipids were extracted with 2 volumes of diethyl ether, the organic phase was withdrawn, residual diethyl ether was removed under a stream of nitrogen, and proteins were precipitated from the extracted aqueous phase as described above.

Western blot analysis with antisera against Erg7p and marker proteins was performed as described by Leber *et al.* (24).

Preparation of an Antiserum against Yeast OSC—To raise an antiserum against yeast OSC, a rabbit was immunized with a peptide of 31,123 Da (Erg7p-459–730) corresponding to the 271 C-terminal amino acids of *S. cerevisiae* Erg7p. The peptide was overexpressed in *Escherichia coli* XL1-blue cells (Stratagene) transformed with a pQE-30 expression vector (Qiagen, Germany) containing 879 bp of the *ERG7* gene. For this purpose, the *ERG7* gene of *S. cerevisiae* strain S288C (*MATα*) (33) was amplified by PCR starting from the chromosomal yeast DNA purified according to the method of Davis *et al.* (34). We used a sense primer 31 bases long, complementary to the antisense strand 42 bases upstream the start codon and an antisense primer 29 bases long, complementary to the sense strand 45 bases downstream the stop codon, including the recognition sequences for *Hin*dIII and *Spe*I (underlined):

sense primer: 5′-CCC AAGCTT ACCTTACCAATAACCGTCGCCC

antisense primer: 5′-GG ACTAGT TAGTTTCTAATTGTTGCAGCC.

In a 50-μl PCR 100 ng of chromosomal DNA was amplified with 0.5 unit of DNA Vent polymerase (New England Biolabs) for 30 cycles (1 min at 95 °C, 1 min at 63 °C, and 3.5 min at 72 °C, each) in the presence of 100 pmol of each primer, 10 nmol of each dNTP, and 2 mM Mg^{2+}. The resulting 2,300-bp PCR product was digested with *Hin*dIII and *Spe*I and ligated into the plasmid pBluescript II KS (Stratagene) digested with the same restriction enzymes. The recombinant plasmid was used

to transform *E. coli* DH5α cells (Invitrogen). The sequence of the cloned gene was determined at BioPark Bioindustry Park Canavese (Colleretto Giacosa, Italy) using the same primers as for PCR; it matched unambiguously the published *ERG7* gene (GenBank accession number U23488).

To amplify the C-terminal part of the *ERG7* gene coding for the peptide Erg7p-459–730 in the expression vector pQE-30, the *ERG7* DNA was transferred from pBluescript II KS to a pUC-18 vector (Amersham Biosciences, Inc.) by cutting both vectors using restriction endonucleases *Kpn*I and *Xba*I. The DNA fragment corresponding to Erg7p-459–730 was excised from pUC-18 with *Pst*I with cleavage sites in the internal sequence of *ERG7* at position 1,373 and in the sequence of the polylinker of pUC-18. The resulting 879-bp fragment was ligated into the pQE-30 polylinker with *Pst*I, in-frame with the ATG and the His_6 tag of the vector. The recombinant vectors were used to transform *E. coli* XL1-blue cells, and the transformed clones were screened for the presence of the gene in the correct orientation by cutting asymmetrically with *Kpn*I and *Spe*I. Clones containing the DNA in the correct orientation showed, as expected, two bands of 0.9 and 3.5 kilobase pairs.

The peptide Erg7p-459–730 was expressed in *E. coli* XL1-blue cells grown on Terrific Broth (TB) containing 0.1 μg/ml ampicillin, 0.0125 μg/ml tetracycline, and 0.4 mM isopropyl-1-thio-β-D-galactopyranoside as inducer. The protein was found in inclusion bodies and solubilized with 1% SDS in 50 mM phosphate buffer (pH 8). The protein was purified further after binding to a Co^{2+} Talon metal chelating resin (CLONTECH) by elution with 50 mM phosphate buffer (pH 8) containing 0.1% SDS and 250 mM imidazole.

Prior to immunization the protein was purified on 10% SDS-PAGE gels, and the band with an apparent molecular mass of 31 kDa was eluted from gel slices with an electroeluter model 422 (Bio-Rad) according to the manufacturer's instructions. This isolate was used to immunize rabbits by standard procedures (31).

Lipid Analysis—Neutral lipids of yeast homogenate and isolated subcellular fractions were extracted according to Folch *et al.* (35). Quantification of ergosteryl esters and triacylglycerols was carried out as described by Leber *et al.* (24).

Nonsaponifiable lipids of homogenate, 30,000 × *g* washed microsomes, and lipid particles were prepared by hydrolyzing 0.5 ml of total lipid extract (containing ~400, 100, and 40 μg of lipid, respectively) with 10% (w/v) methanolic KOH (1 ml) for 1 h at 80 °C. Nonsaponified lipids were extracted three times with 1 ml of diethyl ether. The extracts were combined and dried under a stream of nitrogen. Lipids were dissolved in 1 ml of methanol and analyzed on an HPLC Merck Hitachi with a C_{18} column (5 μm, 250 × 4 mm; Lichrospher 100 RP-18, Merck), equipped with a Shimatzu CBM-10A. The column was eluted with methanol at a flow rate of 0.9 ml/min, and the absorbance of the eluate was monitored at 210 nm. Sterols and sterol precursors were quantified by comparison with standards.

Incorporation of [2-^{14}C]Acetate into Sterols—Sterol biosynthesis in whole yeast cells was followed by incorporation of [2-^{14}C]acetate into nonsaponifiable lipids as described before (36). Briefly, washed cells (10–20 × 10^6 cells) were resuspended in 5 ml of 25 mM KH_2PO_4/NaOH buffer (pH 6.5) containing 1% glucose, 0.1 mg/ml Tween 80, incubated with 2 μCi of [2-^{14}C]acetate (50 mCi/mM) and shaken for 2 h at 30 °C. Then, cells were harvested by centrifugation and saponified in 50% ethanol containing 15% KOH for 2 h at 80 °C. Nonsaponifiable lipids were extracted twice with 1 ml of light petroleum and separated on silica gel plates (Merck) using *n*-hexane/ethyl acetate (85:15; v/v) as developing solvent and authentic standards of ergosterol, lanosterol, dioxidosqualene, oxidosqualene, and squalene. Radioactivity in separated bands was measured using a System 2000 Imaging Scanner (Packard).

OSC Assay—3S-2,3-[^{14}C]Oxidosqualene was prepared by incubating a pig liver S_{10} preparation with [^{14}C]mevalonate as described by Popjak (37), in the presence of 0.1 mM of the OSC inhibitor U14266A (25). After 3 h of incubation the reaction was stopped by adding 1 volume of 15% KOH in methanol, and lipids were saponified at 80 °C for 30 min. The nonsaponifiable lipids were extracted three times with 2 volumes of light petroleum and separated by two-step TLC on silica gel plates (20 × 20 cm, 0.5-mm layer). The plates were first developed in light petroleum to a height of about 10 cm above the origin. After drying, the plates were developed to 15 cm above the origin with *n*-hexane/ethyl acetate (90:10; v/v). Radioactive bands corresponding to 2,3-oxidosqualene were scraped off and eluted with dichloromethane. The extract was brought to dryness under a stream of nitrogen and 3S-2,3-[^{14}C]oxidosqualene was dissolved in benzene.

OSC activity in total yeast homogenate (6 mg of protein/ml), lipid particles (0.03 mg of protein/ml), microsomes (0.5 mg of protein/ml), and cytosol (5 mg of protein/ml) was determined as described elsewhere (36). Briefly, 0.5 ml of the different subcellular fractions were incubated with 3S-2,3-[^{14}C]oxidosqualene (5,000 cpm, 25 μM) in the presence of Tween 80 (0.2 mg/ml) and Triton X-100 (1 mg/ml) in 10 mM MES/Tris (pH 6.9) and 0.2 mM EDTA for 30 min at 35 °C under vigorous shaking in a water bath. The reaction was stopped by adding 1 ml of 15% KOH in methanol, and lipids were saponified at 80 °C for 30 min. The nonsaponifiable lipids were extracted twice with 1 ml of light petroleum and separated on silica gel plates using dichloromethane as developing solvent. Radioactivity in 2,3-oxidosqualene and lanosterol was counted using a System 2000 Imaging Scanner (Packard). The amount of product formed was used for the calculation of the enzyme activity.

TABLE I

Enzymatic activity of OSC in homogenate and subcellular fractions of wild-type FL100 cells grown aerobically or anaerobically

OSC activity was measured in the presence of 1 mg/ml Triton X-100. Results are the means of two separate experiments with duplicate incubations, each. The maximum deviation from the mean was less than 10%. Numbers in parentheses are enrichment factors over the homogenate.

Enzyme source	Specific activity	
	Aerobic growth	Anaerobic growth
	nmol h^{-1} mg^{-1}	
Homogenate	2.96 (1)	2.90 (1)
Lipid particles	686 (232)	265 (91)
30,000 × *g* microsomes	5.11 (1.7)	1.00 (0.3)

RESULTS

Localization of OSC in Yeast Subcellular Compartments—Distribution of OSC activity among subcellular fractions was determined by measuring the enzyme activity in different compartments isolated from a wild-type yeast strain cultivated under aerobic and anaerobic conditions. Results summarized in Table I revealed that OSC activity was 230-fold enriched in lipid particles prepared from aerobically grown cells. This high enrichment over the homogenate resembles typical markers of lipid particles such as steryl esters, triacylglycerols, and Erg6p (Table II). An almost 100-fold enrichment of OSC activity was also observed in lipid particles prepared from anaerobically grown cells. Among the microsomal fractions recovered at multiple *g*-forces, only 30,000 × *g* microsomes, the fraction with the highest enrichment of "classical" microsomal markers (38), contained minor activity (Table I). No activity was detected in other microsomal fractions, in vacuoles, or in the cytosolic fraction (data not shown).

The small but significant activity of OSC in 30,000 × *g* microsomal membrane fraction led us to speculate about the true localization of the enzyme in this compartment. This result deserved our attention insofar as microsomal fractions were considered a good source for yeast OSC (10, 40). To test whether or not the presence of OSC activity in the microsomal pellet was caused by contamination of this fraction with lipid particles, additional washing steps of 30,000 × *g* microsomes prepared from aerobically grown cells were performed assuming that contaminating material would be removed. After each washing step, OSC activity and the content of lipid particle markers were tested. As shown in Fig. 3, the stepwise decrease of triacylglycerol, steryl esters, and Erg6p, typical markers of lipid particles, paralleled the decrease of OSC activity, indicating that the presence of the enzyme in crude 30,000 × *g* microsomes can be largely attributed to the attachment of lipid particles during the fractionation procedure. As a control, Sec61p remained associated with the microsomal fraction during the washing procedure.

Possible activating effects of cytosolic or microsomal factors on OSC activity of lipid particles were tested by addition of a cytosolic fraction or microsomes from an *erg7* deletion strain, which lacks OSC activity, to the assay mixture either in the

TABLE II
Distribution of markers between the endoplasmic reticulum (30,000 × g washed microsomes) and lipid particles

Marker	Enrichment over the homogenate			
	Aerobic growth		Anaerobic growth	
	Endoplasmic reticulum	Lipid particles	Endoplasmic reticulum	Lipid particles
	fold			
Sec61p[a]	3.9	ND	5.0	0.2
BiP[a]	1.8	ND	3.0	0.2
Steryl esters[b]	0.5	981	0.9	116
Triacylglycerols[b]	1.2	544	1.5	101
Erg6p[a]	2.0	1,000	2.0	1,000

[a] Equivalent amounts of protein of each fraction were separated by SDS-PAGE and subjected to Western blot analysis using the respective antiserum. The intensity of the immunological signal in the homogenate was set at 1, and the intensities of signals measured in the endoplasmic reticulum and lipid particles were set in relation. Data were obtained from at least three independent experiments. ND, not detectable.
[b] Amounts of triacylglycerol and steryl esters (mg/mg of protein) in the homogenate were set at 1, and the enrichment in the respective fraction was calculated from the corresponding values. Data were obtained from three independent experiments.

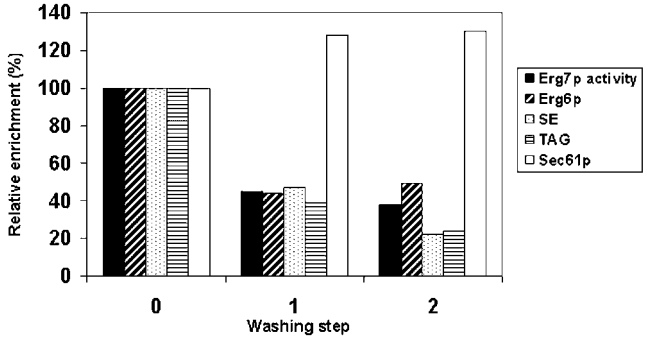

FIG. 3. **Occurrence of residual Erg7p in microsomes.** Endoplasmic reticulum (30,000 × *g* microsomes) prepared by standard methods as described under "Experimental Procedures" was subjected to two additional washes with 10 mM Tris/Cl^- (pH 7.5). OSC distribution was measured by enzymatic activity. Triacylglycerol (*TAG*) and steryl esters (*SE*) were quantified after thin layer chromatographic separation, and Erg6p (sterol Δ^{24}-methyltransferase) and Sec61p were determined by Western blot analysis.

TABLE III
Enzymatic properties of OSC associated with lipid particles

Results are the mean values of two separate experiments with duplicate incubations, each. The maximum deviations from the mean were less than 10%. ND, not detectable. BSA, bovine serum albumin.

Enzyme source[a]	Activation factor[b]
Lipid particles	1.0
Lipid particles + Triton X-100[c]	10.5
Lipid particles + *erg7* microsomes	1.8
Lipid particles + *erg7* microsomes + Triton X-100[c]	7.5
Lipid particles + *erg7* cytosol	2.3
Lipid particles + *erg7* cytosol + Triton X-100[c]	13.2
Lipid particles + BSA	1.5
Lipid particles + BSA + Triton X-100[c]	7.3
erg7 microsomes	ND
erg7 microsomes + Triton X-100[c]	ND
erg7 cytosol	ND
erg7 cytosol + Triton X-100[c]	ND

[a] Protein concentrations: lipid particles, 0.022 mg/ml; microsomes, 1.5 mg/ml; cytosol, 2.5 mg/ml; BSA, 0.75 mg/ml.
[b] The specific activity of OSC in lipid particles in the absence of detergent was set at 1.
[c] The final concentration of Triton X-100 in the assay mixture was 1 mg/ml.

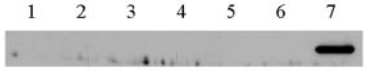

FIG. 4. **Subcellular distribution of Erg7p determined by Western blot analysis.** *Lanes 1* and *2*, 10 μg of homogenate; *lane 3*, 10 μg of 30,000 × *g* washed microsomes; *lane 4*, 10 μg of 40,000 × *g* washed microsomes; *lane 5*, 10 μg of 100,000 × *g* washed microsomes; *lane 6*, 10 μg of vacuoles; *lane 7*, 2 μg of lipid particles.

presence or in the absence of the detergent Triton X-100. Only weak activating effects were observed with the cytosolic fraction and with microsomes of the *erg7* strain (Table III). In all cases OSC present in lipid particles required a detergent for full activity.

As an alternative to the measurement of enzyme activity, Western blot analysis was performed to determine the subcellular distribution of the *ERG7* gene product. As a probe we used a rabbit antibody raised against a 31-kDa fragment of yeast OSC expressed in *E. coli*. Similar to enzymatic assays, Western blot analysis revealed that Erg7p is highly enriched in lipid particles and practically absent from other subcellular fractions (Fig. 4).

Finally, fluorescence microscopic inspection revealed the typical lipid particle staining of Erg7p-GFP (Fig. 5*A*). In contrast, the GFP hybrid of another enzyme involved in ergosterol biosynthesis, Erg4p-GFP, which was used as a control, is located in the endoplasmic reticulum (Fig. 5*B*) as had been shown before by Zweytick *et al.* (41).

Subcellular Distribution of 2,3-Oxidosqualene in Cells Depleted of OSC Activity—To obtain indirect evidence for the presence of OSC in certain subcellular compartments, we measured the subcellular distribution of the substrate of this enzyme, 2,3-oxidosqualene, in wild-type, an *erg7* deletion mutant, and in wild-type treated with an inhibitor of OSC. We assumed that oxidosqualene is at least in part retained in that compartment that lacks the enzyme activity because of the mutation or the action of the inhibitor. 2,3-Oxidosqualene, unlike other intermediates of sterol biosynthesis, is completely absent in lipid extracts from wild-type cultured under standard aerobic conditions (36). Thus, accumulation of this precursor even at very low concentrations can be detected easily. Tracer experiments using [^{14}C]acetate as a precursor demonstrated the absence of 2,3-oxidosqualene and dioxidosqualene from lipid extracts of wild-type cells grown aerobically (Table IV). In the absence of OSC activity, *i.e.* in an *erg7* mutant, both precursors accumulated, and neither lanosterol nor ergosterol was labeled. When wild-type cells were exposed to the OSC inhibitor U14266A (25), a significant amount of the label was also detected in lanosterol and ergosterol, indicating that the block of OSC was incomplete.

The relative distribution of oxidosqualene between the endoplasmic reticulum (30,000 × *g* washed microsomes) and lipid particles was determined by HPLC analysis of nonsaponifiable lipids extracted from the respective fractions. In wild-type cells grown under aerobic conditions, both the endoplasmic reticulum and lipid particles lack oxidosqualene and dioxidosqual-

ene, and the major product of the sterol biosynthetic pathway is ergosterol (Table V). In the *erg7* mutant strain, oxidosqualene and dioxidosqualene accumulated at a huge amount in lipid particles. The occurrence of ergosterol in homogenate and subcellular fractions of *erg7* is because of the supplementation with ergosterol, which is required to maintain growth of this mutant. Efficiency of the OSC activity block, however, is documented by the lack of lanosterol in lipid extracts from *erg7*. Significant amounts of oxidosqualene and dioxidosqualene were also detectable in the endoplasmic reticulum fraction. These precursors were not removed by repeated washes of the 30,000 × *g* membrane pellet and were therefore not attributable to contamination with lipid particles. Similar to the *erg7* mutation, incubation of wild-type cells with the OSC inhibitor U14266A led to accumulation of oxidosqualene in lipid particles and, at a lesser extent, in the endoplasmic reticulum (Table V). Dioxidosqualene was only detectable in the lipid particle fraction of U14266A-inhibited cells. The incomplete block by the inhibitor caused the appearance of lanosterol in the lipid extract of these fractions.

To test the assumption that the substrate of an enzyme accumulates at the site of its blocked enzymatic activity, we determined subcellular distribution of sterol precursors in wild-type and *erg7* cells grown under anaerobic conditions. Anaerobiosis efficiently inhibits squalene epoxidase as demonstrated by accumulation of squalene (Table VI). Similar to an *erg1* mutant, which lacks squalene epoxidase (24), lipid particles of anaerobically grown *erg7* cells were highly enriched in squalene. Minor amounts of oxidosqualene and lanosterol, found in subcellular fractions of the wild-type are the result of incomplete anaerobiosis during spheroplasting and initial steps of cell fractionation. For the same reason, some oxidosqualene was found in lipid particles of the *erg7* mutant. In summary, these data confirm the view that lipid particles of the yeast are an active site of sterol synthesis.

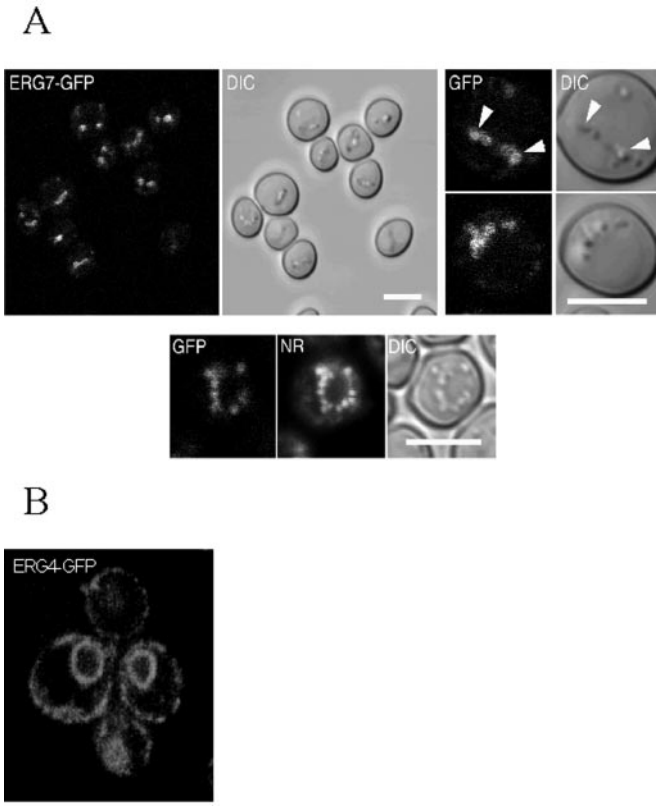

FIG. 5. **Localization of Erg7p-GFP by fluorescence microscopy.** *A*, Erg7p-GFP. Cells expressing ERG7-GFP were grown for 16 h in YPD culture, harvested by centrifugation, and immobilized for microscopy, as described under "Experimental Procedures." *Upper panel*, GFP fluorescence colocalizes with highly light diffractive particles (*arrowheads*), as detected by transmission imaging (*DIC*), characteristic for lipid droplets. *Lower panel*, GFP fluorescence colocalizes with Nile Red fluorescence (*NR*). *Bar* = 5 μm. *B*, Erg4p-GFP.

DISCUSSION

Here we provide evidence that yeast OSC, a key enzyme of sterol biosynthesis, is located mainly in lipid particles, a subcellular compartment previously regarded only as a lipid depot, but now recognized as an organelle endowed with a more complex role in lipid metabolism and trafficking (24, 42, 43). Our conclusion is based on the distribution of enzyme activity in different compartments, Western blot analysis using isolated yeast subcellular fractions, *in vivo* localization of Erg7p-GFP by fluorescence microscopy and the accumulation of oxidosqualene, the substrate of OSC, in lipid particles of cells lacking this enzyme activity. Only minor activity of OSC was detected in the endoplasmic reticulum, which was surprising insofar as microsomes were regarded as a suitable source of OSC in previous studies (10, 40).

Another important result of this work was the observation that the enzymatic activity of OSC is influenced by the detergent:lipid ratio and probably the detergent:protein ratio. Thus, it was important to optimize assay conditions for the enzymatic determination of OSC distribution. Nevertheless, the caveat regarding different environmental effects on the enzyme even *in vitro* remained. Therefore, Western blot analysis using an antiserum against Erg7p and fluorescence microscopic inspection of cells expressing Erg7p-GFP fusion protein were essential supplements to confirm the subcellular localization of OSC. Finally, the distribution of the OSC substrates in subcellular fractions of strains that lack Erg7p, or under conditions of inhibited OSC provided another piece of evidence indicating that lipid particles are the site of active OSC in yeast. The assumption that accumulation of oxidosqualene in lipid particles is a common response to the increased intracellular level of this sterol intermediate is in line with recent data obtained with an *erg27* mutant (kindly provided by M. Bard, Indianapolis), which accumulates squalene, oxidosqualene, and dioxidosqualene (44). Preliminary and unpublished results from our laboratories indicate that in this mutant OSC activity is negligible, and lipid particles are the major site of deposition of oxidosqualene and dioxidosqualene.

TABLE IV
Incorporation of [^{14}C]acetate into nonsaponifiable lipids of wild-type and erg7

Results are the mean values of two separate experiments with duplicate incubations, each. The maximum deviations from the mean were less than 10%; ND, not detectable.

Strain	% total radioactivity incorporated				
	Squalene	Oxidosqualene	Dioxidosqualene	Lanosterol[a]	Ergosterol[a]
FL100 (wild-type)	2.0	ND	ND	13.6	84.4
erg7	2.0	81.0	17.0	ND	ND
FL100 + U14 (0.1 mM)[b]	1.4	51.1	0.4	1.0	46.1

[a] Nonsaponifiable extract contains free sterols and sterols from steryl esters.
[b] U14266A (U14) is an inhibitor of OSC (see "Experimental Procedures").

TABLE V

Sterol and sterol precursor distribution in subcellular fractions of aerobically grown wild-type and erg7 cells

Results are the means of two separate experiments with duplicate incubations, each. The maximum deviations from the mean were less than 10%; ND, not detectable.

Subcellular fraction	Squalene		Oxidosqualene		Dioxidosqualene		Lanosterol[a]		Ergosterol[a]	
	μg/mg	%	μg/mg	%	μg/mg	%	μg/mg	%	μg/mg	%
FL100 homogenate	0.18	0.9	ND		ND		0.05	0.2	20.30	98.9
FL100 lipid particles	25.00	0.5	ND		ND		102	2.0	5,000	97.5
FL100 30,000 × *g* washed microsomes	0.35	0.3	ND		ND		0.01	<0.01	116.71	99.7
erg7 homogenate	0.13	0.6	10.90	47.2	2.50	10.8	ND		9.55	41.4
erg7 lipid particles	16.62	0.1	16,944	74.3	3167	13.9	ND		2,667	11.7
erg7 30,000 × *g* washed microsomes	0.11	0.2	8.07	18.1	3.12	7.0	ND		33.32	74.7
FL100 + U14 (0.1 mM)[b] homogenate	0.36	4.4	2.20	26.9	0.26	3.2	0.10	1.2	5.27	64.3
FL100 + U14 (0.1 mM)[b] lipid particles	50.00	0.3	10,750	72.6	1,000	6.8	1,000	6.8	2,000	13.5
FL100 + U14 (0.1 mM)[b] 30,000 × *g* washed microsomes	0.17	0.4	18.33	46.6	ND		0.83	2.1	20.00	50.8

[a] Nonsaponifiable extract contains free sterols and sterols from steryl esters.
[b] U14266A (U14) is an inhibitor of OSC (see "Experimental Procedures").

TABLE VI

Sterol and sterol precursor distribution in subcellular fractions of anaerobically grown wild-type and erg7 cells

Results are the means of two separate experiments with duplicate incubations, each. The maximum deviations from the mean were less than 10%; ND, not detectable.

Subcellular fraction	Squalene		Oxidosqualene		Dioxidosqualene		Lanosterol[a]		Ergosterol[a]	
	μg/mg	%	μg/mg	%	μg/mg	%	μg/mg	%	μg/mg	%
FL100 homogenate	6.61	14.5	0.25	0.5	ND		0.80	1.7	37.92	83.2
FL100 lipid particles	548.40	50.3	4.31	0.4	ND		21.51	2.0	516.00	47.3
FL100 30,000 × *g* washed microsomes	0.96	2.9	ND		ND		0.16	0.5	32.00	96.6
erg7 homogenate	8.12	21.6	0.64	1.7	ND		ND		28.90	76.7
erg7 lipid particles	840.00	48.6	90.01	5.2	ND		ND		800.00	46.2
erg7 30,000 × *g* washed microsomes	1.67	12.1	ND		ND		ND		12.10	87.9

[a] Nonsaponifiable extract contains free sterols and sterols from steryl esters.

In the present study, as well as in previous work (36), we observed that oxidosqualene, the product of the squalene epoxidase Erg1p, never accumulates in growing yeast cells unless OSC is inhibited. We can speculate that epoxidation of squalene by Erg1p is the rate-limiting step for the transition from acyclic to cyclic intermediates of the sterol biosynthetic route, whereas OSC similar to other nonregulated enzymes may act at very low physiological concentrations of its substrate and thus remove newly formed oxidosqualene rapidly. When OSC is deleted or inhibited, retaining of the bulk of the accumulated oxidosqualene within lipid particles appears to be important for the cell to avoid perturbance of membranes from other organelles.

Localization of OSC, a key enzyme in sterol synthesis, in lipid particles raises the question as to a possible interaction of Erg7p with the squalene epoxidase, Erg1p, which catalyzes the preceding step of sterol biosynthesis. These two enzymes, which utilize or form, respectively, the sterol intermediate oxidosqualene, do not completely colocalize within the yeast cell. Whereas Erg7p appears to be located almost exclusively in lipid particles, Erg1p is distributed among lipid particles and the endoplasmic reticulum. Enzymatic analysis using isolated organelles revealed that the only active form of squalene epoxidase is associated with the endoplasmic reticulum (24), whereas lipid particles harbor a "silent," probably inactive form of the enzyme. It was suggested that a reductase, which is required for the activity of Erg1p, may be absent from lipid particles but present in the endoplasmic reticulum. A certain amount of Erg1p may be deposited on the surface of lipid particles during formation of these droplets from the endoplasmic reticulum and may return to its site of active action when sterol biosynthesis is turned on (24).

The scenario discussed above relies on a complex model that suggests structural and functional relationship of lipid particles and the endoplasmic reticulum. Physical contact between these two compartments appears to be a prerequisite for concerted action of squalene epoxidase, an as yet unidentified reductase, and OSC. This view becomes even more complex by the fact that Erg9p, squalene synthase, is a component of the endoplasmic reticulum.[2] Thus, conversion of the acyclic intermediate farnesyl-pyrophosphate to the first cyclic intermediate of sterol synthesis, lanosterol, is accomplished by (i) synthesis of squalene in the endoplasmic reticulum, (ii) epoxidation of squalene in the endoplasmic reticulum and/or in lipid particles, and (iii) cyclization of oxidosqualene mainly in lipid particles. Interaction of lipid particles with the endoplasmic reticulum is also required during esterification of lanosterol, which is one of the major substrates of the steryl ester synthase Are1p present in the endoplasmic reticulum (45). Furthermore, at least the last step of sterol synthesis in yeast catalyzed by the sterol C-24(28) reductase, Erg4p, is located in the endoplasmic reticulum (41). Thus sterol intermediates have to migrate at some stage from lipid particles to the endoplasmic reticulum to complete the sterol biosynthetic pathway. Finally, steryl esters formed in the endoplasmic reticulum are deposited exclusively in lipid particles. Precise coordination of the metabolic steps involved is required to maintain sterol homeostasis in the cell. Different sensitivity of various fungi and yeasts to inhibitors of late steps of sterol biosynthesis, *e.g.* to allylamines (39, 46), could depend on a different ability of the different species to form such a lipid homeostasis device.

[2] E. Zinser, unpublished results.

The relationship of lipid particles and endoplasmic reticulum may be result of the particles' origin from the endoplasmic reticulum which was discussed not only for yeast (23) but especially for plant cells (for review, see Ref. 42). Regarding the physiological role of lipid particles a picture emerges which ascribes a variety of different functions related to lipid metabolism to this compartment, namely lipid storage, lipid biosynthesis and metabolic conversion, lipid trafficking and probably shielding of membrane-perturbing hydrophobic compounds. The last function was demonstrated in this work, especially by the observation that squalene and its derivatives are stored in lipid particles, when their cellular concentration exceeds the toxicity threshold.

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Yeast Oxidosqualene Cyclase (Erg7p) Is a Major Component of Lipid Particles

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