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Effect of Lipid Particle Biogenesis on the Subcellular Distribution of Squalene in the Yeast *Saccharomyces cerevisiae*[*] [S]

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Squalene belongs to the group of isoprenoids and is a precursor for the synthesis of sterols, steroids, and ubiquinons. In the yeast *Saccharomyces cerevisiae*, the amount of squalene can be increased by variation of growth conditions or by genetic manipulation. In this report, we show that a *hem1*Δ mutant accumulated a large amount of squalene, which was stored almost exclusively in cytoplasmic lipid particles/droplets. Interestingly, a strain bearing a *hem1*Δ deletion in a *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ quadruple mutant background (QM*hem1*Δ), which is devoid of the classical storage lipids, triacylglycerols and steryl esters, and lacks lipid particles, accumulated squalene at similar amounts as the *hem1*Δ mutant in a wild type background. In QM*hem1*Δ, however, increased amounts of squalene were found in cellular membranes, especially in microsomes. The fact that QM*hem1*Δ did not form lipid particles indicated that accumulation of squalene solely was not sufficient to initiate proliferation of lipid particles. Most importantly, these results also demonstrated that (i) squalene was not lipotoxic under the conditions tested, and (ii) organelle membranes in yeast can accommodate relatively large quantities of this non-polar lipid without compromising cellular functions. In summary, localization of squalene as described here can be regarded as an unconventional example of non-polar lipid storage in cellular membranes.

During the last decades, the search for novel drugs and compounds used in biotechnology led to an increased interest in natural products with specific properties. One of the substances detected in biological screenings was squalene, an intermediate in sterol biosynthesis. Squalene is a natural compound that belongs to the group of isoprenoids and is a precursor for the synthesis of sterols, steroids, and ubiquinons. It is one of the most important lipids in skin cells, where it secures beneficial mechanical properties of the skin (*e.g.* skin hydration) (1). Squalene also belongs to the family of antioxidants (2, 3) and was shown to possess antilipidemic and membrane-modulating properties (4, 5). It has also been reported for treatment of skin disorders, cancer, cardiac ailments, and liver diseases (6, 7). Presently, squalene used for commercial purposes is isolated from shark liver oil or olive oil. However, alternative biotechnological systems that might lead to high yield production of this non-polar lipid became attractive. As one of these systems, the yeast *Saccharomyces cerevisiae* was considered.

In the yeast, squalene is synthesized in a sequence of reactions starting from acetyl-CoA, involving a number of steps catalyzed by Erg proteins (8). The squalene synthase Erg9p converts farnesyl diphosphate to squalene in an NAD(P)H-dependent reaction. Erg9p is localized to the endoplasmic reticulum. In the following oxygen-dependent reaction, the squalene epoxidase Erg1p catalyzes formation of 2,3-oxidosqualene (Fig. 1). Erg1p was shown to be dually located within the yeast cell, namely in lipid particles and in the microsomal fraction (9). Interestingly, the enzyme from lipid particles did not exhibit activity *in vitro*, indicating that additional components of the endoplasmic reticulum were required. The next step in the sterol biosynthetic route is catalyzed by the oxidosqualene cyclase (lanosterol synthase) Erg7p, which forms lanosterol, the first sterol in this pathway. Lanosterol is then further converted in several steps to the yeast-specific ergosterol.

The process of sterol biosynthesis is strictly aerobic not only because of the direct oxygen requirement for squalene epoxidation catalyzed by Erg1p but also due to the involvement of heme (synthesized in an oxygen-dependent manner) in several steps in ergosterol biosynthesis. As an example, heme is essential for the activity of the sterol-14-α-demethylase Erg11p (see Fig. 1), an NADPH-heme-dependent cytochrome P450 protein (10, 11). Consequently, yeast cells become auxotrophic for sterols and unsaturated fatty acids when grown anaerobically or under heme deficiency. To rescue cell viability under such conditions, sterols and unsaturated fatty acids have to be supplemented with the growth medium (12–15). When yeast cells are unable to synthesize ergosterol in the absence of oxygen or due to heme deficiency, squalene accumulates. Other strategies to cause a cellular increase of squalene in the yeast are overexpression of *HMG1* (16, 17) and disruption of *ERG1* or *ERG7* (18, 19).

The aim of the present study was to investigate localization of squalene in yeast cells, especially under conditions that stimulated accumulation of this non-polar lipid. For this purpose, we used cells bearing a deletion of the *HEM1* gene in a wild type background. Previous studies from our laboratory (20) had already suggested the presence of squalene in lipid particles. Because lipid particles serve as storage compartment for neu-

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TABLE 1
Yeast strains used in this study

Yeast strains	Genotype	Source
Wild type BY4742	*MATa*; *his3D1*; *leu2D0*; *lys2D0*; *ura3D0*	Euroscarf
QM *dga1*Δ *lro1*Δ *are1*Δ *are2*Δ	*MATa*; *his3D1*; *leu2D0*; *lys2D0*; *ura3D0*; *dga1*::*KanMX4*; *lro1*::*KanMX4*; *are1*::*KanMX4*; *are2*::*KanMX4*	K. Athenstaedt
*hem1*Δ	*MATa*; *his3D1*; *leu2D0*; *lys2D0*; *ura3D0*; *hem1*::*LEU2*	This study
QM*hem1*Δ	*MATa*; *his3D1*; *leu2D0*; *lys2D0*; *ura3D0*; *dga1*::*KanMX4*; *lro1*::*KanMX4*; *are1*::*KanMX4*; *are2*::*KanMX4*; *hem1*::*LEU2*	This study

tral lipids like triacylglycerols (TAG)[2] or steryl esters (SE), this compartment appeared to be the "logical" depot for the excess of squalene as well. In the present work, however, we extended our investigations to the subcellular distribution of squalene in cells deprived of lipid particles. This situation occurs in a *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ quadruple mutant (QM), which lacks the four enzymes catalyzing TAG and SE formation in the yeast (21–23). The QM grows like wild type under standard conditions but is compromised when fatty acids are present in the medium (24, 25) or used as a carbon source.[3]

This cellular scenario of the QM was considered to be highly relevant for possible lipotoxic effects of squalene when accumulated as a consequence of various manipulations. As will be shown in this work, obvious lipotoxic effects of squalene were avoided in a QM*hem1*Δ strain by incorporation of this compound into subcellular membranes. This finding was surprising and has never been described in the literature for biological membranes, although squalene incorporation into artificial membranes has been reported before (26, 27).

EXPERIMENTAL PROCEDURES

Strains, Culture Conditions, and Subcellular Fractionation—Yeast strains used in this study are listed in Table 1. Inactivation of the *HEM1* gene was performed by using the pUC19 plasmid, which contains a *hem1*::*LEU2* disruption cassette. The plasmid was cleaved using the restriction enzymes BamHI and HindIII, and the disruption cassette was transformed into yeast cells by standard procedures. The disruption of *HEM1* in mutants was confirmed by colony PCR.

Cells were grown aerobically to the early or late stationary phase at 30 °C in YPD medium containing 1% yeast extract (Oxoid), 2% peptone (Oxoid), and 2% glucose (Merck). Media were inoculated with precultures to the A_{600} of 0.1. Strains deleted of *HEM1* were grown on YPD medium supplemented with either 50 μg/ml δ-aminolevulinic acid (δALA) or 20 μg/ml ergosterol with 0.06% Tween 80 as a source of oleic acid.

The yeast lipid particle fraction was obtained at high purity from cells grown to the stationary phase, as described by Leber *et al.* (20), with an additional washing step with 9 M urea (28). Isolation of other subcellular fractions (*e.g.* mitochondria, heavy and light microsomes, and plasma membrane) used in this study was described by Zinser and Daum (29). The quality of subcellular fractions was routinely tested by Western blot analysis (see below).

Protein Analysis—Proteins were quantified by the method of Lowry *et al.* (30), using bovine serum albumin as a standard. Polypeptides were precipitated with trichloroacetic acid and solubilized in 0.1% SDS, 0.1 M NaOH prior to quantification. Samples of the lipid particle fractions were delipidated prior to protein quantification. Non-polar 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 as described above.

SDS-PAGE was carried out by the method of Laemmli (31). Samples were denaturated at 37 °C to avoid hydrolysis of polypeptides. Proteins on gels were detected by staining with Coomassie Blue. Western blot analysis was performed as described by Haid and Suissa (32). Proteins were detected by enzyme-linked immunosorbent assay (ELISA) using rabbit antiserum as primary antibody and peroxidase-conjugated goat anti-rabbit IgG as second antibody. Primary antibodies used in this study were from rabbits and directed against yeast Erg6p, Por1p, Wbp1p, and Gas1p. The enhanced chemiluminescent signal detection kit SuperSignal™ (Pierce) was used to visualize immunoreactive bands, which were quantified densitometrically.

Lipid Analysis—Lipids from yeast cells were extracted as described by Folch *et al.* (33). For quantification of neutral lipids, extracts were applied to silica gel 60 plates with the aid of a sample applicator (CAMAG, Automatic TLC Sampler 4, Muttenz, Switzerland), and chromatograms were developed in an ascending manner by a two-step developing system. First, light petroleum/diethyl ether/acetic acid (70:30:2, v/v/v) was used as mobile phase, and plates were developed to half-distance of the plate. Then plates were dried briefly and further developed to the top of the plate using the second mobile phase consisting of light petroleum/diethyl ether (49:1, v/v). To visualize separated bands, TLC plates were dipped into a charring solution consisting of 0.63 g of $MnCl_2 \cdot 4H_2O$, 60 ml of water, 60 ml of methanol, and 4 ml of concentrated sulfuric acid, briefly dried, and heated at 100 °C for 30 min. Then lipids were quantified by densitometric scanning at 400 nm using a Shimadzu dual-wavelength chromatoscanner CS-930 with triolein and cholesteryl ester as standards.

Squalene and individual sterols (free sterols and SE) from whole cells or subcellular fractions were identified and quantified by GLC-MS (34). GLC-MS was performed on an HP 5890 gas chromatograph equipped with a mass-selective detector HP 5972, using an HP5-MS capillary column (30 m × 0.25 mm, 0.25-μm film thickness). Aliquots of 1 μl were injected in the splitless mode at a 270 °C injection temperature with helium as a carrier gas at a flow rate of 0.9 ml/min in constant flow mode. The following temperature program was used: 1 min at 100 °C,

[2] The abbreviations used are: TAG, triacylglycerol(s); SE, steryl ester(s); QM, quadruple mutant; δALA, δ-aminolevulinic acid; GLC-MS, gas/liquid chromatography-mass spectrometry.

[3] M. Spanova, T. Czabany, G. Zellnig, E. Leitner, I. Hapala, and G. Daum, unpublished results.

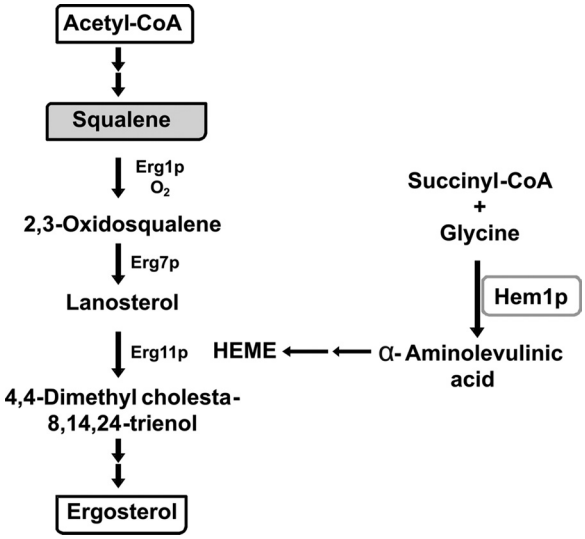

FIGURE 1. **Heme dependence of ergosterol synthesis.** The yeast sterol-14-α-demethylase (Erg11p) is a cytochrome P450 protein and depends on heme. Deletion of *HEM1* can be overcome by supplementing yeast cells with δALA.

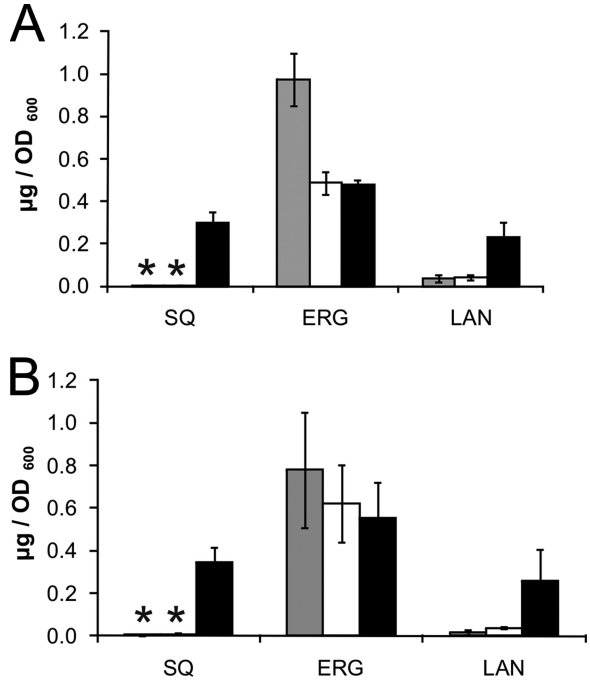

FIGURE 2. **Sterol composition and squalene accumulation in *hem1*Δ mutants.** *A*, wild type BY4742 (*gray bar*), *hem1*Δ + δALA (*white bar*), and *hem1*Δ + ergosterol + Tween 80 (*black bar*). *B*, QM *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ (*gray bar*), QM*hem1*Δ + δALA (*white bar*), and QM*hem1*Δ + ergosterol + Tween 80 (*black bar*). Cells were grown to the stationary phase, lipids were extracted, and sterols/squalene were quantified by GLC-MS. Data are mean values of five independent experiments. *Error bars*, S.D. values. *, in these samples, only marginal amounts of the respective compound were detected. *SQ*, squalene; *ERG*, ergosterol; *LAN*, lanosterol.

10 °C/min to 250 °C, and 3 °C/min to 310 °C. Mass spectra were acquired in the scan mode (scan range 200–550 atomic mass units) with 3.27 scans/s. Sterols were identified based on their mass fragmentation pattern.

Fluorescence Microscopy of Lipid Particles—Nile Red staining was performed as described by Greenspan *et al.* (35). Microscopic pictures were taken with an Olympus BX50 photomicroscope equipped with a color digital camera DP70 (Olympus) using a mirror unit U-MSWGZ (dichroic mirror, DM570; exciter filter, BP480-550; barrier filter, BA590). Nile Red fluorescence of lipid particles was detected at an emission wavelength of 590 nm. Digital color pictures were transformed to gray scale figures by standard software.

Electron Microscopy of Yeast Cells—For ultrastructural inspection, cells were grown under aerobic conditions at 30 °C on YPD with or without supplementations as described above. Cells were harvested in the early stationary phase by centrifugation and washed three times with double-distilled water. Subsequently, cells were fixed for 5 min in a 1% aqueous solution of $KMnO_4$ at room temperature, washed with distilled water, and fixed in a 1% aqueous solution of $KMnO_4$ for 20 min again. Fixed cells were washed three times in distilled water and incubated in 0.5% aqueous uranylacetate overnight at 4 °C. Samples were dehydrated in a graded series of acetone (50, 70, 90, and 100%) and gradually infiltrated with increasing concentrations of Spurr resin (30, 50, 70, and 100%) mixed with acetone for a minimum of 3 h for each step. Samples were finally embedded in pure, fresh Spurr resin and polymerized at 60 °C for 48 h. Ultrathin sections of 80 nm were stained with lead citrate and viewed with a Philips CM 10 electron microscope.

RESULTS

Heme-deficient Cells Accumulate Squalene—Disruption of *HEM1* encoding δ-aminolevulinic acid synthase leads to cessation of the ergosterol pathway at the level of lanosterol demethylation (see Fig. 1). Consequently, *hem1*Δ mutants accumulated lanosterol (Fig. 2*A*) but also showed an ~300-fold increase of squalene. This accumulation of squalene resulted in a marked change of the neutral lipid pattern. Quantification of neutral lipids after TLC separation revealed that in wild type, the ratio of squalene to SE and TAG, respectively, was close to zero, whereas it increased to 1.7 (for SE) and 0.88 (for TAG) in the *hem1*Δ mutant. It has to be noted that ergosterol (free and acylated form) detected in *hem1*Δ strains was derived either from exogenous sterol supplementation or through bypassing the defect by supplementation of δALA to the growth medium (14, 15), which restored heme synthesis (see Fig. 2*A*). Under the latter conditions, the sterol pattern was close to wild type grown with or without supplements (data not shown), and squalene accumulation was not observed.

The QM *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ, which lacks enzymes required for the biosynthesis of TAG and SE, exhibited almost the same sterol pattern as wild type (Fig. 2*B*). *HEM1* deletion in the QM background led also to a huge increase of squalene. Because QM*hem1*Δ was unable to synthesize TAG and SE, the accumulated squalene encountered for the major non-polar lipid species in this strain. This observation led us to investigate whether or not this large amount of squalene was sufficient and capable of initiating the formation of lipid storage particles.

Squalene Alone Does Not Initiate Lipid Particle Formation—As described above, squalene accumulated in heme-deficient cells. The question remained where squalene was localized under these conditions. In wild type cells, a small amount of squalene (0.5% of total mass) has been detected in lipid particles (20, 36). Therefore, it was conceivable that squalene accumu-

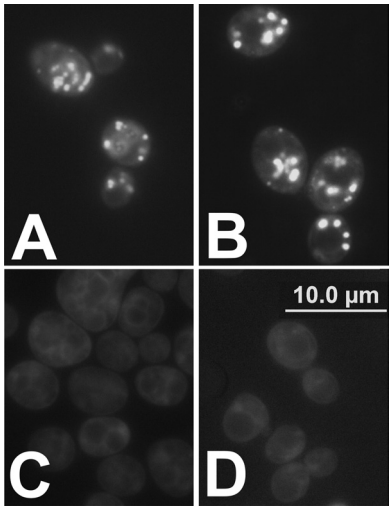

FIGURE 3. **Visualization of lipid storage compartments by fluorescence microscopy.** Wild type BY4742 (*A*), *hem1*Δ (*B*), QM (*C*), and QM*hem1*Δ (*D*) were grown to the stationary phase, stained with Nile Red, and inspected by fluorescent microscope as described under "Experimental Procedures." *Bar*, 10 μm.

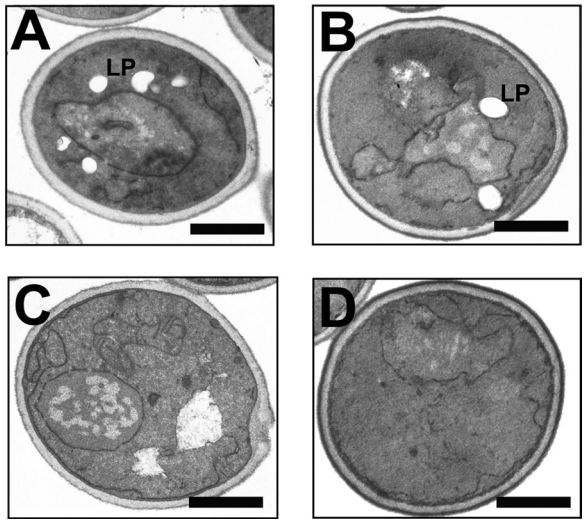

FIGURE 4. **Overproduction of squalene in a quadruple mutant *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ does not induce lipid particle formation.** Wild type (*A*), *hem1*Δ (*B*), QM (*C*), and QM*hem1*Δ (*D*) were grown to the stationary phase and then processed and inspected by electron microscopy as described under "Experimental Procedures." *Bar*, 1 μm. *LP*, lipid particle.

lating in a *hem1*Δ mutant would also be preferentially found in this lipid storage compartment. Since lipid particles have been considered as a depot for excess fatty acids that may be toxic for the cell (37), we anticipated that the same storage mechanism may also apply to squalene.

To address this question, we performed fluorescent microscopy of cells stained with the fluorescent dye Nile Red. This dye is specific for neutral lipids and has been widely used to visualize lipid particles/droplets. As expected, lipid particles were observed in wild type and in a *hem1*Δ deletion mutant (Fig. 3, *A* and *B*). Thus, the usual lipid storage compartment was present in these cells, but the localization of squalene could not be attributed by this method. In accordance with published observations, lipid particles were missing in QM (Fig. 3*C*). The striking result, however, was that in the squalene-accumulating QM*hem1*Δ strain, lipid particles or lipid droplet-like structures were not detected either (Fig. 3*D*). This result could be interpreted in two different ways. First, lipid particles may indeed not be formed in QM cells even under conditions promoting squalene accumulation. Second, squalene and consequently squalene-loaded lipid particles might not be properly visualized with Nile Red. To address the latter question, we subjected the same cells to electron microscopy. As can be seen from Fig. 4, the results paralleled completely the fluorescence microscopy analysis. Although lipid particles were detected in wild type and *hem1*Δ, no such structures were observed in the QM background. Most notably, the QM*hem1*Δ strain did not contain lipid particle structures, indicating that squalene accumulation in the absence of TAG and SE was not sufficient to induce lipid particle proliferation. This view was supported by the fact that all our attempts to isolate lipid particles from strains with QM background failed. These results together with data shown in Fig. 2 also suggested that under these conditions, squalene was obviously localized to other subcellular sites.

*Subcellular Distribution of Squalene in Wild Type and in the Quadruple Mutant dga1*Δ*lro1*Δ*are1*Δ*are2*Δ—To obtain a deeper insight into the subcellular distribution of squalene in the different strains mentioned above, mitochondria, 30,000 × *g*, 40,000 × *g*, and 100,000 × *g* microsomes, lipid particles, plasma membrane, and cytosol were isolated from wild type, *hem1*Δ, QM, and QM*hem1*Δ and subjected to detailed analysis.

First, all isolated subcellular fractions were tested for purity by Western blotting. Enzyme markers for plasma membrane (Gas1p), mitochondria (Por1p), microsomes (Wbp1p), and lipid particles (Erg6p) were used. As shown in Table 2, all organelles from the different strains were obtained at the expected quality in line with previously published results (29). In all strains, plasma membrane and lipid particles were obtained at high enrichment, and mitochondria and microsomal fractions were obtained at good quality with acceptable cross-contamination. It must be mentioned, however, that in *hem1*Δ and QM*hem1*Δ contamination between microsomes and mitochondria was higher than in wild type and QM. This problem could not be overcome by a number of modifications in the isolation protocol and appears to be an intrinsic property of these organelles under heme depletion.

The subcellular distribution of squalene and ergosterol in wild type and mutant strains is shown in Table 3, and the enrichment of these components in the individual organelles from the different strains is documented in supplemental Figs. S1–S4. In wild type, the highest amount of ergosterol was detected in microsomal and mitochondrial compartments (see Table 3), although the highest enrichment was found in the plasma membrane (supplemental Fig. S1*A*). Squalene was present mainly in lipid particles and microsomes. The observation that squalene accumulated in lipid particles from wild type (106 mg/g organelle protein; see supplemental Fig. S1*B*) was in line with previous findings (20, 36). In the lipid particle fraction, ergosterol, lanosterol, and other sterol precursors (not shown) were also enriched. It has to be noted that the large majority of sterols in lipid particles occurs in the acylated form as SE.

In the QM, sterols were found mainly in mitochondria and microsomes (see Table 3 and supplemental Fig. S2). It is note-

TABLE 2

Quality control of yeast subcellular fractions

For growth conditions and the isolation of subcellular compartments, see "Experimental Procedures." The relative enrichment of markers in the homogenate was set as 1. Marker proteins were as follows: Gas1p (β-1,3-glucanosyltransferase (marker for plasma membrane)); Wbp1p (β-subunit of the oligosaccharyl transferase glycoprotein complex (marker for microsomes)); Por1p (porin (marker for mitochondria)); Erg6p (Δ(24)-sterol C-methyltransferase (marker for lipid particles)). ND, not detectable.

	Relative enrichment													
	Wild type				*hem1*Δ				QM			QM*hem1*Δ		
	Gas1p	Wbp1p	Por1p	Erg6p	Gas1p	Wbp1p	Por1p	Erg6p	Gas1p	Wbp1p	Por1p	Gas1p	Wbp1p	Por1p
	-fold				*-fold*				*-fold*			*-fold*		
Mitochondria	ND	1.0 ± 0.66	3.8 ± 1.5	ND	ND	1.0 ± 0.40	3.2 ± 0.91	0.6	ND	1.5 ± 0.75	2.3 ± 0.43	2.0	2.1 ± 0.043	2.0 ± 0.50
Microsomes, 30.000 × *g*	ND	2.5 ± 0.38	0.5 ± 0.2	ND	ND	1.3 ± 0.24	0.1 ± 0.09	0.4	ND	3.0 ± 0.40	ND	1.0	2.9 ± 0.96	1.2 ± 0.20
Microsomes, 40.000 × *g*	ND	1.9 ± 0.075	ND	ND	ND	1.1 ± 0.24	ND	ND	ND	2.8 ± 0.96	ND	ND	1.1 ± 0.53	0.2 ± 0.058
Microsomes, 100.000 × *g*	ND	0.7 ± 0.001	ND	ND	ND	0.3 ± 0.2	ND	ND	ND	ND	ND	ND	ND	0.1 ± 0.08
Cytosol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plasma membrane	~100	1.2 ± 0.14	0.3 ± 0.07	ND	~100	2.5 ± 1.1	2.7 ± 1.2	ND	~100	2.3 ± 1.1	2.5 ± 0.78	~100	1.1 ± 0.66	1.2 ± 0.75
Lipid particles	ND	1.5 ± 0.75	0.5 ± 0.3	~100	ND	1.0 ± 0.60	0.5 ± 0.3	~100						

TABLE 3

Quantification of squalene and sterols in organelles

Cells were cultivated to the stationary phase with supplements, organelles were isolated as described by Zinser *et al.* (29), proteins were quantified as described by Lowry *et al.* (30), and sterols were analyzed by GLC-MS. ND, not detectable.

	Percentage of homogenate									
	Wild type		*hem1*Δ			QM		QM*hem1*Δ		
	Squalene	Ergosterol	Squalene	Ergosterol	Lanosterol	Squalene	Ergosterol	Squalene	Ergosterol	Lanosterol
	%	%	%	%	%	%	%	%	%	%
Mitochondria	ND	4.28 ± 2.28	3.07 ± 1.18	6.31 ± 2.60	8.14 ± 1.43	27.56 ± 2.78	27.70 ± 5.89	17.66 ± 5.18	16.59 ± 4.51	13.55 ± 0.64
Microsomes, 30.000 × *g*	24.79 ± 16.66	11.45 ± 2.09	9.06 ± 5.04	39.80 ± 15.67	41.85 ± 10.17	33.42 ± 13.75	33.41 ± 12.54	42.28 ± 5.44	46.64 ± 0.69	41.59 ± 11.59
Microsomes, 40.000 × *g*	7.90 ± 4.59	8.76 ± 2.19	7.03 ± 1.40	22.75 ± 14.32	25.29 ± 5.96	32.14 ± 15.05	33.45 ± 15.45	26.69 ± 8.70	29.27 ± 5.07	30.19 ± 12.18
Microsomes, 100.000 × *g*	ND	7.39 ± 3.38	5.35 ± 2.64	21.00 ± 18.89	19.72 ± 7.30	5.81 ± 1.02	2.94 ± 0.35	10.81 ± 5.18	4.87 ± 2.07	12.13 ± 9.24
Cytosol	ND	0.38 ± 0.02	0.67 ± 0.24	1.68 ± 0.55	1.24 ± 0.30	0.82 ± 0.83	0.95 ± 0.28	2.03 ± 0.78	1.25 ± 0.36	2.06 ± 0.12
Plasma membrane	0.45 ± 0.31	1.35 ± 0.23	0.15 ± 0.05	1.00 ± 0.05	0.95 ± 0.64	0.25 ± 0.03	1.56 ± 0.02	0.54 ± 0.26	1.38 ± 0.28	0.47 ± 0.31
Lipid particles	66.86 ± 3.11	66.40 ± 2.50	74.68 ± 0.37	7.47 ± 4.78	2.81 ± 2.30					

worthy that the enrichment of ergosterol in the plasma membrane was not as high as in wild type, whereas larger concentrations of ergosterol were detected in microsomes and also in mitochondria. Due to the quadruple deletions of *DGA1*, *LRO1*, *ARE1* and *ARE2*, lipid particles were missing in this strain. Similar to ergosterol, squalene was found in microsomal and mitochondrial membranes of the QM. The enrichment of squalene in microsomes and mitochondria from the QM was higher than in wild type but still moderate. The slight accumulation of squalene in QM may be the result of reduced formation of squalene epoxidase (Erg1p) in this strain (23).

*Subcellular Distribution of Squalene in hem1*Δ *and in QMhem1*Δ—Yeast strains deleted of *HEM1* bear defects in the sterol biosynthetic pathway and fatty acid desaturation. To maintain growth of such mutants, the respective supplements in the media need to be provided (see "Experimental Procedures"). As a consequence of this defect, *hem1*Δ mutants formed substantial amounts of squalene and lanosterol (see Fig. 2). Based on previous findings (20, 36), we assumed that under these conditions, large amounts of squalene accumulated in lipid particles. As can be seen from Table 3, this was correct. Although in other subcellular membranes, ergosterol (imported from the medium) was still the major sterol, and squalene was present only at smaller concentrations (supplemental Fig. S3*A*), the level of squalene in lipid particles of *hem1*Δ by far exceeded that of ergosterol (supplemental Fig. S3*B*). This finding was not surprising because of the low esterification efficiency of external ergosterol, as reported by Valachovic *et al.* (38).

In Fig. 2, it is shown that a QM deleted of *HEM1* accumulated squalene at similar amounts as *hem1*Δ in the wild type background. Because the QM*hem1*Δ strain did not contain lipid particles (see Figs. 3 and 4), it was assumed that squalene might be localized to other organelles. Indeed, marked amounts of squalene were detected in mitochondrial and microsomal fractions (see Table 3 and supplemental Fig. S4). This subcellular distribution of squalene reflected very much the localization of ergosterol and lanosterol in these cells. Due to the cross-contamination of mitochondria and microsomes (see Table 2) in QM*hem1*Δ compared with QM and wild type, the absolute amount of squalene and sterols present in these fractions must be interpreted with caution. However, the fact remains that under heme depletion in a QM background, intracellular membranes were able to accommodate large amounts of the nonpolar lipid squalene.

DISCUSSION

For the work presented here, we created a situation in the cell caused by accumulation of squalene that we considered lipotoxic. For this purpose, we used the yeast *S. cerevisiae* bearing a deletion of the *HEM1* gene. Such *hem1*Δ strains have been widely used as a model for anaerobic growth (39, 40), because lack of cytochromes results in metabolic changes similar to oxygen deficiency. It has, however, also been shown that

such *hem1*Δ strains can grow reasonably well as long as ergosterol and unsaturated fatty acids are supplied from the medium (14, 15).

In the present study, we show that squalene overproduced under heme deficiency in wild type cells mainly ends up in lipid particles. This was expected because this compartment is considered a depot or even a sink for storage lipids produced in excess (*e.g.* fatty acids). The real challenge for the yeast cell arises when lipid particles are missing. This situation occurs in a heme-deficient quadruple mutant QM*hem1*Δ. To our surprise, these cells were viable although accumulating substantial amounts of squalene, indicating that squalene was not lipotoxic, at least under the conditions chosen and at the amounts accumulated.

We considered the findings described above very important for two more reasons. (i) Squalene, similar to TAG and SE, is formed in the endoplasmic reticulum (41). In previous studies from our laboratory (22, 23), we had found that synthesis of either TAG or SE was sufficient to promote the biosynthesis of lipid particles. A model widely advocated for lipid particle biogenesis suggests that neutral lipid synthesized in the endoplasmic reticulum may be the sparking point for lipid particle biogenesis (22). This may, however, be true for TAG and SE but, according to our results, not for squalene. (ii) Because the QM*hem1*Δ mutant strain did not contain lipid particles but nevertheless accumulated large amounts of squalene, this component had to be accommodated in organelle fractions different from lipid particles. As already observed in the *hem1*Δ mutant strain in a wild type background, squalene of QM*hem1*Δ was detected in mitochondria and microsomes but at much higher amounts.

Localization of squalene in biological membranes existing mainly as phospholipid bilayers is obviously not a regular situation because, due to its shape, the high hydrophobicity, and the lack of a polar head group, squalene cannot be expected to form bilayer structures *per se*. However, localization of squalene in the membranes may be explained by the observations of Lohner *et al.* (26), who studied the influence of squalene on artificial membranes. These authors concluded that (i) squalene at a concentration of 6 mol % in phospholipid vesicles changed the lamellar-to-inverse hexagonal phase transition by increasing the size of the tubes of the inverse hexagonal phase, and (ii) squalene must be accommodated in a disordered region of the bilayer, suggesting that squalene exists in a coiled rather than extended conformation and localizes to the interior of the bilayer. The localization of polyisoprene hydrocarbons in the midplane of a lipid bilayer was also reported by Hauss *et al.* (27). These authors studied the role of squalane (the saturated form of squalene) in artificial phospholipid membranes by neutron diffraction. They argued that this specific membrane structure may function as a proton permeability barrier (42). This may also apply to biological membranes, because yeast cells accumulating squalene in membranes appear to exhibit altered sensitivity to low pH and high salt concentrations compared with wild type.[3]

The question remains how squalene synthesized in the endoplasmic reticulum reaches its different cellular destinations. In the case of storage in the lipid particles, a co-migration with TAG and SE in the course of lipid particle biogenesis may be anticipated. Transport of squalene to other organelles and incorporation into membranes may involve other mechanisms generally discussed for lipid transport and assembly such as protein-mediated transport, vesicle flux, or membrane contact (43, 44). In summary, subcellular distribution of squalene, as shown in this study, can be regarded as a novel facet of lipid storage in membranes and shows at the same time the high flexibility of the yeast as a model system to adapt to lipid stress situations.

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