Alteration in membrane fluidity and lipid composition, and modulation of H⁺-ATPase activity in *Saccharomyces cerevisiae* caused by decanoic acid

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Decanoic acid, a lipophilic agent, inhibited *in vitro* the plasma membrane H⁺-ATPase of *Saccharomyces cerevisiae* grown in YPD medium. Conversely, when decanoic acid (35 µM) was present in the growth medium, the measured H⁺-ATPase activity was four times higher than that of control cells. K_m and pH and orthovanadate sensitivity were the same for the two growth conditions, which indicated that H⁺-ATPase activation was not due to conformational changes in the enzyme. The activation process was not entirely reversible which showed that plasma membrane H⁺-ATPase activation is due to several mechanisms. 1,6-diphenyl-1,3,5-hexatriene anisotropy performed on protoplasts from cells grown in YPD revealed that as decanoic acid concentration was increased, anisotropy significantly decreased, i.e. membrane fluidity increased. Cells grown in media containing decanoic acid exhibited greater membrane fluidity compared with control cells. Furthermore, these cells did not show any fluidifying effect when increased concentrations of decanoic acid were added. Chemical analysis of cell membrane lipid composition revealed a modification in the distribution of the phospholipid fatty acids and sterols in cells grown in the presence of 35 µM decanoic acid compared with control cells. Our results support the view that the plasma membrane H⁺-ATPase activation induced by decanoic acid is correlated with an alteration in membrane lipid constituents.

Keywords: *Saccharomyces cerevisiae*, fluorescence anisotropy, membrane lipid composition, H⁺-ATPase, decanoic acid

INTRODUCTION

Medium-chain fatty acids (octanoic and decanoic acid) are produced during alcoholic fermentation (Lafon-Lafourcade *et al.*, 1984). The amount of these fatty acids released into the fermentation medium is dependent on the yeast strain, medium composition and fermentation conditions (temperature, pH, aeration) (Jones *et al.*, 1981; Krauss & Forch, 1975).

Medium-chain fatty acids as antimicrobial components have been described by Nordström (1964) and Freeze *et al.* (1973). At concentrations of up to 114 and 46 µM of octanoic and decanoic acid, respectively, the specific growth of *Saccharomyces cerevisiae* decreased as an exponential function of the fatty acid concentration (Viegas *et al.*, 1989). These fatty acids also increased the specific

thermal death of *Saccharomyces bayanus* (Sá-Correia, 1986), and stimulated ethanol-induced leakage of amino acids and 260 nm-absorbing compounds from *S. cerevisiae* (Sá-Correia *et al.*, 1989).

Although octanoic and decanoic acid toxicity is well known, the primary site of inhibition and the mechanism of action have not yet been unambiguously determined. Their action is tightly dependent on the medium pH: the toxic effect increases as pH decreases, indicating that the undissociated molecule is the toxic form, decanoic acid being more inhibitory than octanoic acid (Viegas *et al.*, 1989). It has been proposed that the undissociated form, which is soluble in membrane phospholipids, enters the microbial cell across the plasma membrane by passive diffusion. The presence of such compounds inside the plasma membrane is expected to modify membrane organization (Ingram & Buttke, 1984), thus affecting membrane-bound enzymes such as those involved in transport systems.

Abbreviation: DPH, 1,6-diphenyl-1,3,5-hexatriene

The plasma membrane H^+ -ATPase is responsible for the creation of an electrochemical gradient which constitutes the driving force for nutrient transport (Malpartida & Serrano, 1981; Goffeau & Slayman, 1981). Viegas & Sá-Correia (1991) reported an *in vivo* activation of the plasma membrane H^+ -ATPase of *S. cerevisiae* by octanoic acid, which was supposed to counteract increased cytoplasmic acidification induced by subsequent exposure to octanoic acid. This hypothesis was supported by a recent study in which Stevens & Servaas Hofmeyr (1993) showed that lower-chain-length fatty acids exerted their effect by acting as proton carriers across the yeast plasma membrane, thereby reducing the intracellular pH and disrupting the proton gradient. Decanoic acid, for example, enhanced the passive H^+ influx across the plasma membrane.

Lipophilic compounds such as octanoic and decanoic acids might also disrupt the plasma membrane (Suomalainen & Nurminen, 1976). Compounds which can be incorporated into lipids often have an effect on the lipid composition of micro-organisms grown in their presence (Rose, 1989). It is therefore possible that H^+ -ATPase activation is due to changes in the lipid environment. We have investigated this possibility by studying the effect of decanoic acid on the activity of plasma membrane H^+ -ATPase in cells grown either with or without decanoic acid in relation to membrane fluidity and lipid composition.

METHODS

Yeast strain, growth media and culture conditions. *S. cerevisiae* 3079 is a commercial wine yeast selected by the Bureau Interprofessionnel des Vins de Bourgogne (21000 Beaune, France). Cells were grown in 500 ml flasks in 250 ml YPD medium (0.5%, w/v, Difco yeast extract; 1%, w/v, peptone; 2%, w/v, glucose; pH 6). Ethanol solutions of decanoic acid were added to this medium, the final ethanol concentration being 0.5% (v/v). Ethanol allowed decanoic acid solubilization in the medium. Flasks were inoculated (1×10^6 cells ml^{-1}) with stationary-phase-cultures and incubated at 25 °C with shaking (160 r.p.m.). Cellular growth was followed by measuring OD_{620} . The specific growth rate was calculated by least-squares fitting to the linear part of semi-exponential (OD_{620}) plots versus time. For dry weight determinations, samples of yeast cell suspension (5 ml) were centrifuged at 1500 *g* for 10 min, the pellet was resuspended in water and collected by centrifugation on a pre-dried, weighed cellulose acetate filter (0.45 μm). The wet filter was dried in a microwave oven to constant weight. For all experiments, cells were harvested at mid-exponential phase of growth. This was determined by following the time-course of growth. At this point, the pH of both media, with or without decanoic acid, was 5.2.

Activity of plasma membrane H^+ -ATPase. Cells were harvested by centrifugation (1500 *g*, 10 min, 4 °C). Yeast plasma membranes used for enzyme activity determination were prepared as described by Goffeau & Dufour (1988). For protein determination, a portion (20 μl) of plasma membrane preparation was solubilized by boiling for 5 min after addition of 980 μl 0.5 M NaOH. The protein content was then assayed by using the Lowry method with BSA as a standard.

The assays for H^+ -ATPase activity were carried out by incubation of the plasma membrane (25–50 μg protein) at 35 °C (the optimum temperature for enzyme activity, Goffeau &

Slayman, 1981) in a final volume of 1 ml containing 6 mM ATP, 12 mM $MgCl_2$, 50 mM MES/NaOH, pH 6 (the optimum pH for enzyme activity) and 10 mM NaN_3 . To study the effects of decanoic acid on the *in vitro* activity of plasma membrane H^+ -ATPase, membrane fractions extracted from cells grown with or without decanoic acid were incubated in the H^+ -ATPase assay mixture (pH 6) for 5 min with increasing concentrations of decanoic acid. Total decanoic acid concentrations were in the range 0–116 μM of the undissociated form. For calculation of the concentrations of the undissociated form, we used a pK_a of 4.9 (Freeze *et al.*, 1973). At pH 6 the undissociated form represents 7.3% of total decanoic acid. Stock solutions of decanoic acids were prepared at pH 11–12 in dilute sodium hydroxide (0.1 M). The pH was then reduced to 6 causing a decanoic acid colloid to be formed. This solution was then dispersed into the assay medium to reach the desired concentration.

Plasma membrane fluidity measurement. Fluidity measurements were performed on protoplasts produced from cells grown either in YPD or YPD plus decanoic acid as described previously (Alexandre *et al.*, 1994a). Membrane fluidity was assessed by measuring fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH), following incorporation into the plasma membrane of protoplasts. A Kontron spectrofluorimeter (model SFM 25) with fixed excitation and emission polarization filters was used to measure fluorescence intensity parallel and perpendicular to the polarization phase of the exciting light. DPH was excited at 355 nm and emission was measured at 440 nm. The measured fluorescence intensities were corrected for background fluorescence and light scattering from the unlabelled sample. The degree of fluorescence anisotropy, *r*, was calculated according to:

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2G \cdot I_{VH}} \quad G = \frac{I_{HV}}{I_{HH}}$$

I_{VV} and I_{VH} are the fluorescence intensities determined at vertical and horizontal orientations of the emission polarizer, when the excitation polarizer is set in the vertical position. I_{HV} and I_{HH} are the fluorescence intensities determined at vertical and horizontal positions of the emission polarizer when the excitation polarizer is set horizontally. *G* is a correction factor for background fluorescence and light scattering. The protoplast suspension was diluted to obtain a maximum A_{440} of 0.15. Twenty microlitres 0.6 mM DPH in tetrahydrofuran was added to 3 ml protoplast suspension containing 1 M sorbitol and the desired decanoic acid concentration. The suspension was incubated at 25 °C $\pm$ 0.5 °C (the growth temperature), with continuous stirring of the sample.

Extraction and analysis of lipids. Lipid extraction was performed as previously described (Alexandre *et al.*, 1994b). Phospholipids were obtained by TLC on plates of kieselgel G60 (0.25 mm thick) and then developed in a solvent system of light petroleum (boiling point 40–60 °C)/diethyl ether/acetic acid (70:30:1, by vol.). The phospholipid band was scraped off and methylated according to Rose & Veazey (1988). GC separation of methyl esters of fatty acids involved a Chrompack apparatus (capillary column, free fatty acid phase; 25 m $\times$ 0.52 mm); column temperature 120–250 °C at 2 °C min^{-1} . The injector port was at 250 °C; the detector port at 300 °C; the carrier gas (N_2) pressure was 40 kPa.

The analysis of total sterols requires saponification of lipid extract by using ethanolic KOH at 33% (w/v). A known amount of epicoprostanol (5 μg) as internal standard was added before saponification. Sterols were extracted and trimethylsilyl (TMS) esters were prepared according to Gambert *et al.* (1979).

A Packard 427 gas chromatograph equipped with an OV1 capillary column and flame-ionization detector was used. Separation of TMS derivatives was carried out by programming the column temperature from 160 °C to 350 °C at 2 °C min⁻¹. The flow rate of the carrier gas (N₂) was 1 ml min⁻¹. Authenticity of the identified components was based on known compounds and verified by GC-MS.

RESULTS AND DISCUSSION

Effect of decanoic acid on yeast growth

Addition of increasing concentrations of decanoic acid to the growth medium (pH 6) depressed both specific growth rate and biomass yield (Table 1). The duration of growth latency (lag phase) increased with increasing decanoic acid concentrations (data not shown). The specific growth rate was reduced by 50 % when 35 µM decanoic acid (undissociated form) was present in YPD medium (pH 6). This decrease in biomass in the presence of decanoic acid was expected, since Freeze *et al.* (1973), Hunkova & Fencel (1977), Lafon-Lafourcade *et al.* (1984) and Viegas *et al.* (1989) had already reported a decrease in

biomass yield when another medium-chain fatty acid, octanoic acid, was present in the growth medium. Cole & Keenan (1987) observed that weak acids and hydrogen ions induced a decrease in the growth rate of *Zygosaccharomyces bailii*. Decanoic acid is thought to act as an uncoupler, affecting the electrochemical gradient and the pH_i (Stevens & Servaas Hofmeyr, 1993). The yeast plasma membrane H⁺-ATPase has been implicated in the maintenance of intracellular pH (Serrano, 1988). Therefore we assessed the activity of the plasma membrane H⁺-ATPase of cells grown with or without decanoic acid at 35 µM, the concentration required to reduce the specific growth rate by 50 %.

Plasma membrane H⁺-ATPase activity of cells grown without decanoic acid

Addition of decanoic acid to the H⁺-ATPase assay medium inhibited H⁺-ATPase activity in a concentration-dependent manner (Table 2). The presence of 58 µM decanoic acid (undissociated form) at pH 6 caused 45 % inhibition of H⁺-ATPase activity. It is noteworthy that H⁺-ATPase activity decreased drastically compared with the slight inhibition observed by Viegas & Sá-Correia (1991) with octanoic acid at concentrations of up to 357 µM. This large decrease in H⁺-ATPase activity with lower concentrations of decanoic acid could explain the greater toxicity of decanoic acid compared with octanoic acid. This agrees with the higher liposolubility of the longer chain-length acid (Suomalainen & Nurminen, 1976). According to De Meis (1989), hydrophobic molecules would compete with P_i and inhibit activity of enzymes involved in energy transduction because they partition from the hydrophilic assay medium into the hydrophobic environment of the catalytic site.

Plasma membrane H⁺-ATPase activity of cells grown with decanoic acid

Measured H⁺-ATPase activity of cells grown in the presence of 35 µM decanoic acid revealed an activated state of the enzyme. Specific activity of the H⁺-ATPase was fourfold greater than the activity of control cells (Table 2). The existence of an activated H⁺-ATPase when cells were grown with decanoic acid revealed that

Table 1. Effect of increasing decanoic acid concentrations on the growth of *S. cerevisiae* 3079 grown in YPD (pH 6)

Data are the means of at least four independent experiments.

Growth parameter	Decanoic acid concn (µM)				
	0	12	23	35	46
µ _{max} (h ⁻¹)	0.28	0.24	0.20	0.14	0.11
Doubling time (h)	2.27	2.89	3.46	4.95	6.30
Biomass yield (mg ml ⁻¹)*	1.5	1.4	1.1	0.7	0.5
Time (h)/OD ₆₂₀ †	12/1.2	13/1	14/0.8	18/0.6	24/0.3
Inhibition (%)‡	0	17	28.6	50.5	60.7

* Determined in mid-exponential phase of growth.

† Time at which mid-exponential phase was reached and corresponding OD₆₂₀.

‡ Calculated from the decrease in µ_{max} compared to the control treatment without decanoic acid.

Table 2. Specific activity [in µmol P_i min⁻¹ (mg protein)⁻¹] of the plasma membrane H⁺-ATPase extracted from cells of *S. cerevisiae* 3079 grown in YPD medium with or without decanoic acid, in the presence of increasing decanoic acid concentrations

Data are the means of three independent experiments.

Growth medium	Decanoic acid concn (µM)							
	0	12	23	35	46	58	87	116
YPD	0.54 ± 0.1	0.5 ± 0.1	0.4 ± 0.06	0.4 ± 0.12	0.35 ± 0.1	0.3 ± 0.1	0.2 ± 0.05	0.18 ± 0.1
YPD + 35 µM decanoic acid	2.5 ± 0.2	2.4 ± 0.3	2.2 ± 0.4	2.2 ± 0.2	2.2 ± 0.1	2.2 ± 0.3	1.7 ± 0.3	0.9 ± 0.2

Fig. 1. Characteristics of the plasma membrane H⁺-ATPase of *S. cerevisiae* 3079 grown in YPD (■) or YPD plus 35 μM decanoic acid (□). (a) pH dependence of H⁺-ATPase activity; (b) double reciprocal plots of the effect of ATP concentration on plasma membrane H⁺-ATPase activity; (c) inhibition of plasma membrane H⁺-ATPase by orthovanadate 100% activity 0.55 and 2.3 $\mu\text{mol P}_i \text{ min}^{-1} (\text{mg protein})^{-1}$ for cells grown in YPD and YPD plus decanoic acid, respectively].

physiological changes occurred which constitute a response of the cell to decanoic acid stress. The stimulation of the H⁺-ATPase was not due to conformational changes in the enzyme since the kinetic parameters K_m , and pH and orthovanadate sensitivity, were the same for the two growth conditions (Fig. 1). Viegas & Sá-Correia (1991) reported an activated state of the H⁺-ATPase in the case of cells cultivated with octanoic acid, but the activity was

only 1.5-fold higher than the basal one. It is likely that H⁺-ATPase activation counteracts the cytoplasm acidification due to proton influx movement induced by decanoic acid or intracellular dissociation of this acid (external pH in mid-exponential phase of growth was 5.2). This difference could be explained by the observations of Stevens & Servaas Hofmeyr (1993). These authors reported that the enhancing effect of decanoic acid on passive H⁺ influx was more pronounced than that of octanoic acid.

Reversion of decanoic-acid-induced activation

When cells grown with decanoic acid were incubated for 20 min in YPD medium without decanoic acid, there was a 24% decrease in H⁺-ATPase activity [1.8 ± 0.1 versus $2.3 \pm 0.1 \mu\text{mol P}_i \text{ min}^{-1} (\text{mg protein})^{-1}$; means $\pm$ SD, $n = 4$]. However, the activity did not reach the basal level shown by cells grown in YPD medium alone [$0.55 \pm 0.15 \mu\text{mol P}_i \text{ min}^{-1} (\text{mg protein})^{-1}$], remaining 3.2-fold higher. This indicated that plasma membrane H⁺-ATPase activation induced by decanoic acid is due to a number of potential mechanisms. The activation process of the yeast plasma membrane H⁺-ATPase previously reported differ in several characteristics. Activation by glucose is a reversible process that occurs through changes in V_{max} , affinity to ATP, optimum pH and K_i for orthovanadate of the enzyme (Serrano, 1983). Acidification of the external medium induced an increase of the H⁺-ATPase activity which results from an effect on V_{max} , but in this case the process is irreversible (Eraso & Gancedo, 1987). Activation by nitrogen starvation is also an irreversible process (Benito *et al.*, 1992). All these differences in kinetic parameters of the activation processes could be explained by the existence of different functional domains which interact with the regulatory domain of the H⁺-ATPase (Eraso & Portillo, 1994). The mechanism of activation observed with octanoic, decanoic acid or ethanol could not apparently be attributed to effects on H⁺-ATPase gene expression (Monteiro *et al.*, 1993). One of the mechanisms might occur post-translationally, as suggested by Rao *et al.* (1993) in the case of glucose activation. A recent study reported that changes in intracellular pH affected hydrolytic activities and that protons may regulate catalytic activity by binding to an enzyme region distinct from the transport site. Alternatively, binding of protons to the transport site may activate the catalytic cycle (Feirreira-Peireira *et al.*, 1994). If this hypothesis is correct, it is possible that protons generated by the dissociation of decanoic acid in the cell are responsible in part for the observed activated H⁺-ATPase. In addition, this could explain why, after removal of the acid, part of the activation was lost.

Another possible mechanism of activation could be via alteration of plasma membrane lipid composition. Lipids are essential for the catalytic activity of many membrane-bound enzymes. As allosteric effectors, lipids usually activate the enzyme. The physical state of the bilayer including surface charge, density and fluidity can influence enzyme activity in a number of ways (Gennis, 1989). Large amounts of a substrate incorporated into the bilayer would undoubtedly influence the physical state of the

Table 3. Effect of decanoic acid concentration on fluorescence anisotropy of DPH incorporated into the plasma membrane of *S. cerevisiae* 3079 protoplasts

Data are given as r values and are the means $\pm$ SD of four independent experiments. Values followed by different letters are significantly different according to the Student's t -test ($P < 0.005$).

Medium	Decanoic acid concn (μ M)						
	0	23	35	46	58	87	116
YPD	0.161 $\pm$ 0.002 ^a	0.160 $\pm$ 0.002	0.148 $\pm$ 0.003 ^b	0.146 $\pm$ 0.002	0.140 $\pm$ 0.002	0.138 $\pm$ 0.003	0.137 $\pm$ 0.003
YPD + 35 μ M decanoic acid	0.139 $\pm$ 0.004	0.139 $\pm$ 0.003	0.138 $\pm$ 0.004	0.138 $\pm$ 0.003	0.130 $\pm$ 0.005	0.131 $\pm$ 0.003	0.127 $\pm$ 0.006

membrane. To assess this possibility we measured plasma membrane fluidity by fluorescence anisotropy and determined the lipid composition of cells grown with or without decanoic acid.

Measurement of fluorescence anisotropy

Protoplasts were prepared immediately prior to each experiment. Membranes were labelled with a fluorescent probe and fluorescence anisotropy was measured at 25 °C, the temperature at which cells were cultivated. Changes in anisotropy of DPH following the addition of decanoic acid are shown in Table 3. As decanoic acid concentration increased, DPH anisotropy of the plasma membrane of cells grown in YPD medium was significantly decreased, i.e. membrane fluidity increased. The decanoic acid effect was concentration-related. Different behaviour was observed when fluorescence anisotropy measurements were carried out on cells grown in the presence of 35 μ M decanoic acid (Table 3). These cells exhibited a higher plasma membrane fluidity compared with control cells, with a significantly lower r value. These results led us to propose that the membranes of decanoic-acid- grown cells have been modified. The increase in membrane fluidity is consistent with increased H⁺-ATPase activity, but that activation may also be an adaptive response to lowered pH_i induced by decanoic acid.

No significant changes occurred in *S. cerevisiae* grown with decanoic acid when this acid was added to the protoplast suspension (Table 3). We could not determine from these experiments whether, under these conditions, cells were insensitive to the fluidizing effect of decanoic acid or if the measured anisotropy value reflects the lowest level of membrane fluidity that any concentration of decanoic acid can induce. It is possible that a modification of the plasma membrane lipid composition is responsible for the observed changes in membrane fluidity.

Cell lipid composition

Chemical analyses of the lipid composition of the cells were performed to attempt to explain the observed alteration in fluidity. The percentage of ergosterol and lanosterol from cells grown in medium containing decanoic acid were approximately twice that of control cells.

Table 4. Effect of decanoic acid on sterol composition of *S. cerevisiae* 3079

Data are the means of four independent experiments $\pm$ SD.

Sterol	Percentage of total sterols	
	YPD	YPD + 35 μ M decanoic acid
Squalene	1.5 $\pm$ 0.1	0.5 $\pm$ 0.1
Zymosterol	31.4 $\pm$ 0.9	9.8 $\pm$ 0.2
Ergosterol	40.0 $\pm$ 0.7	77.7 $\pm$ 1.7
Fecosterol	10.0 $\pm$ 0.12	4.0 $\pm$ 0.4
Episterol	6.3 $\pm$ 0.3	4.6 $\pm$ 0.3
Ergosta-5,7-dien-3 β -ol	9.3 $\pm$ 0.2	2.0 $\pm$ 0.3
Lanosterol	1.0 $\pm$ 0.5	2.3 $\pm$ 1.0

Table 5. Effect of decanoic acid on fatty acid composition of phospholipids of *S. cerevisiae* 3079

Data are the means of four independent experiments $\pm$ SD.

Fatty acid	Percentage of total fatty acids	
	YPD	YPD + 35 μ M decanoic acid
C16:0	31.7 $\pm$ 1.4	26.0 $\pm$ 0.3
C16:1	31.6 $\pm$ 4.3	9.4 $\pm$ 0.2
C18:0	20.8 $\pm$ 0.2	20.3 $\pm$ 0.3
C18:1	15.8 $\pm$ 1.5	44.1 $\pm$ 0.6

At the same time a marked decrease in other sterols was observed (Table 4).

Also affected was the fatty acid composition of yeasts grown with decanoic acid. Of these fatty acids, the proportion of C16:1 decreased considerably (-70%) and that of C18:1 increased by 65% (Table 5). The modifications in sterol and fatty acid composition of the cell could be responsible for the changes observed in plasma membrane fluidity.

Our experiments have shown that *S. cerevisiae* appears to adopt at least two different strategies to cope with the decanoic acid effect. One of these mechanisms is direct proton extrusion through the plasma membrane H^+ -ATPase, as is indicated by the activation of this enzyme when cells were grown in the presence of decanoic acid. A second mechanism involves a modification of plasma membrane organization. It is likely that these modifications are partly responsible for the existence of an activated state of the H^+ -ATPase, although the possibility remains that acid-induced reduction in pH_i may also play a regulating role. Our study did not allow us to distinguish between bulk lipid effects and specific allosteric effects but previous studies support the view that lipids could potentially activate the H^+ -ATPase (Martinez-Cortina *et al.*, 1992). Besides the fact that the plasma membrane H^+ -ATPase has an absolute requirement for lipid in order to function (Serrano *et al.*, 1988), enzyme activity is a function of the type of lipid. The role of sterols is of particular interest in this respect as it has been demonstrated that changes in membrane sterol composition appear to affect the activity of H^+ -ATPase in plants (Cooke & Burden, 1990; Cooke *et al.*, 1993). Moreover, Serrano *et al.* (1988) observed that delipidated H^+ -ATPase reconstituted into phospholipid vesicles showed activation by all phospholipids tested.

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