Activation of plasma membrane ATPase of *Saccharomyces cerevisiae* by octanoic acid

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Plasma membrane ATPase activity of *Saccharomyces cerevisiae* IGC 350711 grown in the presence of the lipophilic acid octanoic acid [4–50 mg l⁻¹ (0.03–0.35 mM), pH 4.0] was 1.5-fold higher than that in cells grown in its absence. The $K_m$ for ATP, the pH profile and the sensitivity to orthovanadate of the basal and the activated forms of the membrane ATPase were identical. This activation was closely associated with a decrease in the biomass yield and an increase in the ethanol yield, and was rapidly reversed *in vivo* after removal of the acid. However, the activated level was preserved when membranes were extracted and subjected to manipulations which eliminated or decreased octanoic acid incorporation in the plasma membrane. The activity of the basal plasma membrane ATPase in the total membrane fraction was slightly increased by incubation at pH 6.5 with octanoic acid at 100 mg l⁻¹ or less (2.4 mg acid form plus 97.6 mg octanoate ion l⁻¹). However, destruction of the permeability barrier between the enzyme and its substrate could not explain the *in vivo* activation. A role for plasma membrane ATPase activation in the regulation of the intracellular pH (pH$_i$) of cells grown with octanoic acid was not proven.

Introduction

Octanoic acid is a highly toxic byproduct of yeast alcoholic fermentation (Sá-Correia, 1986; Viegas et al., 1989). Most antimicrobial food additives are lipophilic acids, and octanoic acid is used in cheese wrappers (Freeze et al., 1973). At concentrations up to 16 mg l⁻¹ (0.11 mM) – the range produced during yeast alcoholic fermentation (Viegas et al., 1989) – octanoic acid decreases maximum specific growth rate and biomass yield, and delays the onset of growth in both *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* (Viegas et al., 1989). As medium pH decreases from 5-4 to 3-0, the toxic effects increase, indicating that the undissociated molecule is the toxic form (Viegas et al., 1989). This undissociated form is highly soluble in membrane phospholipid, and is thought to enter the cell by passive diffusion across the plasma membrane (Eliasz et al., 1976; Warth, 1989). The presence of octanoic acid in the plasma membrane is expected to decrease the membrane's spatial organization (Eliasz et al., 1976; Ingram & Buttke, 1984), resulting in increased membrane permeability, and in non-competitive inhibition of membrane-bound enzymes such as those involved with transport (Freeze et al., 1973; van Uden, 1985). Cytoplasmic enzymes might also be adversely affected by a decrease in the internal pH (pH$_i$) caused by the dissociation of octanoic acid ($pK_a$ 4.9) in the cytoplasm (pH$_i$ ~ 7-0) (Cole & Keenan, 1987; Pampulha & Loureiro-Dias, 1989; Krebs et al., 1983). The intracellular accumulation of ions at low external pH might underly the toxicity of octanoic acid, but dissipation of the transmembrane proton gradient also appears to be important.

The proton-pump ATPase in the plasma membrane of fungi couples ATP hydrolysis to the expulsion of protons, generating a proton gradient (Serrano, 1984). The resulting transmembrane electrochemical gradient drives secondary transport and regulates the activity of many pH-sensitive intracellular enzymes (Serrano, 1984, 1988). Near-maximum ATPase activity is rare, because it is energetically expensive to maintain, and activity is usually much lower (Serrano 1984, 1988). For example, when S. cerevisiae cells are incubated with glucose, ATPase activity increases by as much as 10-fold (Serrano, 1983). The activity is also increased *in vivo* by low pH; this appears to constitute a mechanism for internal pH regulation (Eraso & Gancedo, 1987). The ATPase is not only essential for, but also rate-limiting to, cell growth (Serrano, 1988).
Since the plasma membrane ATPase is a critical target for lipophilic drugs, alteration of the membrane structure could be among the mechanisms of octanoic acid toxicity. Ethanol, for example, affects transmembrane proton flow in *S. cerevisiae* (Cartwright et al., 1986, 1987; Mishra & Prasad, 1988), an effect attributed either to stimulation of the passive proton influx by non-specific increase in plasma membrane permeability (Leão & van Uden, 1984; Salgueiro et al., 1989) or to the inhibition of plasma membrane ATPase as observed in *vivo* (Cartwright et al., 1987). This paper describes the effect of octanoic acid on the growth kinetics and energetics of *S. cerevisiae* IGC 35071III, and compares plasma membrane ATPase activity in cells grown in the presence and absence of octanoic acid.

**Methods**

*Strain, growth media and culture conditions.* The respiratory mutant of *S. cerevisiae* strain IGC 35071III (Sá-Correia et al., 1989; Salgueiro et al., 1988; Viegas et al., 1989) was used. Cells were cultured in liquid medium (pH 4.0) prepared in 0.1 M-citric acid/0.2 M-NaHPO₄ buffer containing 30 g glucose 1⁻¹ and Yeast Nitrogen Base (Difco) at a final concentration of 0.5 mm Na₂HPO₄ buffer. Ethanol solutions of octanoic acid were added to this medium to give a final concentration of 0.5% v/v ethanol. Ethanol allowed octanoic acid solubilization in the aqueous acidic medium without significantly affecting the specific growth rate (Viegas et al., 1989). Cells were grown at 30 °C, with orbital shaking, in conical flasks with needle-perforated rubber bungs. Media were inoculated with standardized liquid inocula (Viegas et al., 1989), the initial biomass being 140 mg dry weight 1⁻¹.

**Measurements of ethanol, glucose and biomass.** Samples were collected by centrifugation and analysed for glucose using the dinitrosalicylic acid method (Miller, 1959). Ethanol was analysed by gas chromatography, using a Hewlett-Packard 5710A gas chromatograph with a flame ionization detector and a Chromosorb WHP (80–100 mesh) column treated with 10% Carbowax 20M. The temperature of both detector and inlet was 250 °C, and the oven operated at 110 °C. Butanol was used as the internal standard. Growth was followed by measuring the OD₆₀₀, and biomass at the stationary phase was determined as cell dry weight.

**Activity of plasma membrane ATPase.** Cells were harvested by centrifugation (4 °C) in the mid-exponential phase when the biomass reached 0.5 mg dry weight ml⁻¹ (OD₆₀₀ = 1.5). The pellets were then resuspended in their supernatants to a cell density of 15 mg dry weight ml⁻¹. After the addition of concentrated solutions of Tris, EDTA and diithiothreitol to final concentrations of 100, 5 and 2 mM respectively (Eraso & Gancedo, 1987), the suspensions were rapidly frozen at −70 °C.

After thawing, cell suspensions (2 ml) were vortexed in eight bursts of 1 min each, interspersed with 1 min on ice, using 1.5 ml glass beads (Sigma, 0.5 mm diam.). The total membrane fraction preparation was obtained as described by Serrano (1983) and yielded 53–70 μg protein (mg dry wt)⁻¹. Protein concentrations in the total membrane fraction were obtained by the method of Bradford (1976) using bovine serum albumin (Sigma) as a standard, and ranged from 8 to 10 mg ml⁻¹.

ATPase activity of the total membrane fraction (80–100 μg protein per 500 μl of the assay mixture) was determined in an assay medium containing 50 mM-MES (pH 5.7 adjusted with 2.5 M-Tris), 10 mM-MgSO₄, 50 mM-KCl, 5 mM-sodium azide (to inhibit mitochondrial ATPase) and 50 mM-KNO₃ (to inhibit vacuolar ATPase) (Serrano, 1983). Under these conditions ATPase activity could be attributed predominantly (87.5–90%) to plasma membrane ATPase. After 5 min incubation at 30 °C, the assay was started by adding ATP (Sigma) (final concn 2 mM). Samples (500 μl) were taken at 2 min intervals and the reaction stopped by adding 500 μl 10% (w/v) TCA. The membranes were removed by centrifugation, and 500 μl 0.8 M-HCl containing 0.5% ammonium molybdate was added to the supernatant. Inorganic phosphate (Pi) liberated was determined as described by Fiske & Subbarow (1925), using Na₂HPO₄, H₂O as standard. ATPase activity was expressed in units of nmol Pi, released min⁻¹ (mg protein)⁻¹.

Kinetic properties for plasma membrane ATPase were determined using the total membrane fraction. *Kₐ* for ATP was calculated by least-squares fitting to a Lineweaver–Burk plot ([ATP] in the range 0.2–40 μM). The pH dependence was measured in the range 4–7.5 (buffers were prepared by the addition of 2 M-Tris to MES).

Orthovanadate inhibition was evaluated by incubating total membrane fractions with assay medium containing Na₂VO₄ (up to 300 μM) at pH 5.7, 30 °C, for 5 min prior to assay. Orthovanadate interfered with the measurement of P, liberation, and different calibrations for Pi were used for each concentration of orthovanadate.

To determine the effect of octanoic acid, membrane fractions were incubated in the assay mix at 30 °C, with octanoic acid, at pH 6.5 (adjusted with 2.5 M-Tris), for 15 min prior to assay. Total octanoic acid concentrations were in the range 0–2000 mg l⁻¹. concentrations of the undissociated form were in the range 0–48 mg l⁻¹. All samples contained 0.5% ethanol.

**Intracellular pH.** The pH of cells was determined from the relative distribution of [2-¹⁴C]propionic acid (Rottenberg, 1979; Pampulha & Loureiro-Dias, 1989). Cells were incubated with octanoic acid either in the growth medium or in citrate/phosphate buffer (pH 4.0). When the OD₆₀₀ had reached 1.5, samples (10 ml) were transferred to 50 ml Erlenmeyer flasks, and octanoic acid was added. These mixtures were incubated with orbital agitation at 30 °C for 0.5 or 47.5 min before the addition of 4 μl 94 mM[2-¹⁴C]propionic acid (Amersham, 1960 MBQ mmol⁻¹). The cell suspensions were immediately incubated at 30 °C for 12.5 min (necessary for the propionic acid to equilibrate between the extracellular medium and the cytoplasm). They were then filtered through a glass fibre filter (Whatman GFC, 25 mm diam.), washed with cold water (10 ml) and their radioactivity measured in a Beckman LS 5000TD scintillation counter. To determine total extracellular propionic acid, 500 μl cell suspension was centrifuged for 1 min, and the radioactivity of 20 μl of the supernatant measured. For buffered media, pH 4.0, the external pH did not vary significantly during incubation. To calculate total propionic acid concentration inside the cells, the internal cell volume was assumed to be constant at a mean value of 1.55 μl (mg dry wt)⁻¹ (Pampulha & Loureiro-Dias, 1989).

**Results and Discussion**

**Kinetics of growth with octanoic acid**

The addition of up to 50 mg octanoic acid l⁻¹ to *S. cerevisiae* IGC 35071III cells in buffered growth medium (pH 4.0; 92% of octanoic acid was present as the undissociated toxic form) depressed both the specific growth rate (from 0.29 to 0.095 h⁻¹) and the biomass yield [from 0.08 to 0.035 g dry wt (g glucose)⁻¹] (Figs 1 and 2). The ethanol yield in batch culture, however, increased from 0.36 to 0.42 g ethanol (g glucose)⁻¹ (Figs 1 and 2).
and 2). In related studies, Warth (1988) reported that for *Zygosaccharomyces bailii*, benzoic acid reduces the growth yield and biomass production, but increases the specific fermentation rate. An uncoupling of *S. cerevisiae* was recently confirmed in experiments in which acetate or propionate were added to the medium in anaerobic glucose-limited chemostat cultures (Verduyn et al., 1990).

Activity of plasma membrane ATPase in cells grown with octanoic acid

Because lipophilic compounds affect transmembrane proton flow, a fundamental prerequisite for growth under acid conditions, or in the presence of lipophilic acids, appears to be the regulation of pH$_i$ (Cole & Keenan, 1987; Eraso & Gancedo, 1987; Warth, 1988).

The activation of plasma membrane ATPase, both in *S. cerevisiae* (Eraso & Gancedo, 1987; Serrano, 1984, 1988) and in *Streptococcus faecalis* (Kobayashi et al., 1984, 1986), has been proposed as a mechanism for regulating pH$_i$ (Serrano, 1984, 1988). Therefore, the activity of plasma membrane ATPase was compared in yeast cells grown in the presence and absence of octanoic acid.

The specific activity of plasma membrane ATPase in crude membrane fractions prepared from cells grown in the presence of octanoic acid was higher than that in membranes isolated from cells grown without it (Fig. 3a). When the cells were harvested, the pH of the medium (3.95-4.00), the glucose consumed (residual glucose 20-25 g l$^{-1}$) and the ethanol produced (1-1.2%, v/v) were all consistent between cultures, despite the presence of up to 30 mg octanoic acid l$^{-1}$ (Fig. 1). At 50 mg octanoic acid l$^{-1}$, the pH was 3.85, residual glucose was 15 g l$^{-1}$ and ethanol 1.5% (v/v) (Fig. 1). Under these standardized conditions, and despite the cells being in batch culture, changes in the plasma membrane ATPase activity were mainly related to the presence of octanoic acid.

The activation of ATPase could be reversed within 10 min by incubating cells in fresh medium without octanoic acid (Fig. 3b). Cells to be used for the total membrane extraction were centrifuged and concentrated in their own growth media, as this prevented reversal of the activation. The activity did not reach basal level (Fig. 3b), and after 120 min incubation, plasma membrane ATPase specific activity increased slightly because of cell growth. The specific activity increased during later exponential growth (unpublished results). In spite of the rapid *in vivo* reversion of the activated plasma membrane ATPase, the activated state was essentially maintained when membranes were extracted and their incorporated octanoic acid decreased or eliminated. *In vivo* reversal of plasma membrane ATPase activation has also been
The H+-ATPase of the plasma membrane has vital cell functions. This suggests that its activity should be tightly regulated and should depend on the stage of growth, and on the acidity and glucose concentration of the medium (Eraso & Gancedo, 1987; Eraso et al., 1987; Tudury et al., 1985). According to a model proposed by Kobayashi et al. (1984, 1986), when S. faecalis is grown in the presence of protonophores or in acid media, the pH, is regulated by cytoplasmic pH-dependent changes in the amount and activity of the H+-ATPase. The synthesis of S. faecalis H+-ATPase is stimulated by low cytoplasmic pH (Kobayashi et al., 1986). A comparison of several characteristics of the plasma membrane ATPases prepared from S. cerevisiae cells grown with and without octanoic acid (30 mg l⁻¹) showed that the pH profiles, \( K_m \) for ATP (3-78 ± 0.01 mM) and orthovanadate sensitivity were identical for the basal and the activated activities (Fig. 4). This suggests that a conformational change was unlikely to have occurred. Stimulation of ATPase biosynthesis, therefore, appeared to be an adequate explanation for our results, as the activated ATPase and the basal ATPase appear to be the same enzyme; moreover, the activated state was preserved after plasma membrane purification. To test the possibility that the observed activation depends on the stimulation of protein synthesis we compared the activity of cells grown in the absence of octanoic acid and incubated for 1 and 2 h with and without octanoic acid, and with and without cycloheximide. These experiments were inconclusive, because activity was higher when cells were harvested slightly later in growth, and growth kinetics were altered by octanoic acid (results not shown).

Effects of octanoic acid on the activity of plasma membrane ATPase in the total membrane fraction

Detergents, in a critical range of concentrations, increase the saturation velocity \( V \) of the S. cerevisiae ATPase in vesicles, with or without a change in the \( K_m \) of the enzyme for ATP (Monk et al., 1989). Moreover, data obtained with the Ca²⁺-ATPase and the Na⁺/K⁺-ATPase suggested that the solvent structure at the catalytic site is involved in energy transduction in biological membranes (Meis, 1989). We therefore tried to elucidate the effect on ATPase activity in the crude membrane fraction, extracted from cells grown with or without octanoic acid, of up to 50 mg l⁻¹ octanoic acid at pH 6.5 (50 mg acid form, plus 1950 mg octanoate ion l⁻¹) are the expected intracellular concentrations of the two forms based on cytoplasmic accumulation; \( pH_i = 6.5 \). Although basal ATPase activity increased slightly when incubated at pH 6.5 with 2-4 mg of the acid form of octanoic acid l⁻¹ (plus 97-6 mg octanoate ion l⁻¹) (Fig. 5),

reported in S. cerevisiae on removal of another inducer, glucose (Serrano, 1983) and in S. faecalis when the low extracellular pH is increased (Koboyashi et al., 1986). The rapid in vivo reversion of the octanoic-acid-induced activation indicates that the activation cannot be attributed mainly to differences in lipid environment of ATPase in slowly growing cells (Hunter & Rose, 1972).

Interestingly, the increase in ATPase activity in cells grown in octanoic-acid-supplemented media (Fig. 3a) paralleled the decrease in growth yield and the concomitant increase in ethanol yield (Fig. 2), suggesting that these phenomena might be related. The specific activity of plasma membrane ATPase decreased slightly in cells grown with concentrations of octanoic acid above 30 mg l⁻¹ (Fig. 3a). It is possible that under the experimental conditions used the ATPase was inhibited by >50 mg l⁻¹, resulting in impaired growth (Serrano, 1988).
Octanoic acid and yeast plasma membrane ATPase

Fig. 4. Comparison of characteristics of plasma membrane ATPase in the total membrane fraction from cells grown in the absence (O) or presence (●) of octanoic acid (30 mg l⁻¹). (a) pH profiles; (b) activity as a function of ATP concentration (Lineweaver-Burke plot); (c) sensitivity to orthovanadate. Results are the means of at least two enzyme assays.

The possible destruction of a permeability barrier between the enzyme and its substrate (Monk et al., 1989) did not explain the in vivo ATPase activation. In fact, the activity of the plasma membrane ATPase extracted from cells grown with 30 mg octanoic acid l⁻¹ was significantly higher than the maximum activity extracted from control cells (grown without octanoic acid) incubated with octanoic acid in vitro. Moreover, no increase in the activity of ATPase activated in vitro was detected after incubation with octanoic acid. Incubation of ATPase with concentrations of total octanoic acid of 100–2000 mg l⁻¹ slightly decreased activity.

The internal pH of cells grown with octanoic acid

The decrease of pHₐ to values which are inhibitory to various key enzymes such as hexokinase and phosphofructokinase (Krebs et al., 1983) is an important mechanism for the deleterious effects of low external pH and weak acids (e.g. benzoic, sorbic, acetic) on yeasts (Krebs et al., 1983; Cole & Keenan, 1987; Pampulha & Loureiro-Dias, 1989). In Rhizobium meliloti, regulation of pH appears to be a fundamental prerequisite for growth under acid conditions (O’Hara et al., 1989), and activation of plasma membrane ATPase is involved in maintenance of pH during growth of S. cerevisiae on acid media (Eraso & Gancedo, 1987). We therefore studied the role of plasma membrane ATPase activation in the regulation of pHₐ in cells grown with octanoic acid. A small decrease in pHₐ was expected in these cells (Fig. 6) due to the very small acid concentrations used; it is therefore unreasonable to suppose that the observed pH drop from 7·1 to 7·0 in cells incubated with 4 mg octanoic acid l⁻¹ (pH 4·0) underlies the ATPase activation. Moreover, there was no difference in the pHₐ decrease in cells incubated with octanoic acid, either in growth medium or in glucose-free buffer, for different periods of time. However, the cells incubated in buffer (pH 4·0) for the longer period had the lowest pHₐ, since no energy source was available to maintain the pH gradient. The pHₐ was also estimated in cells grown with increasing...
concentrations of octanoic acid, taking into consideration the decrease of intracellular volume with the increase in population doubling time, and using the relationship reported by Cole & Keenan (1987). Again, no significant differences in pH_i were detected between cells grown with and without octanoic acid (results not shown).

**Octanoic acid toxicity and plasma membrane ATPase**

Mechanisms proposed for the inhibitory effects of octanoic acid on yeasts must take into account its high liposolubility. In contrast with other less liposoluble fatty acids (acetic, benzoic, propionic, sorbic) (Cole & Keenan, 1987; Krebs et al., 1983; Pampulha & Loureiro-Dias, 1989), concentrations of octanoic acid that severely affected the specific growth rate of *S. cerevisiae* had a negligible effect on pH_i. Owing to its very high lipid–buffer partition coefficient, the concentration of the acid form of octanoic acid in the plasma membrane might become high enough to affect the activity of membrane-bound enzymes. However, the plasma membrane ATPase was not significantly affected when the membrane fraction was incubated with octanoic acid at up to 50 mg l^-1 (acid form) and up to 1950 mg l^-1 (octanoate ion), which might be reached inside the cell under the conditions used. It is noteworthy that although the plasma membrane ATPase was activated in vivo over the whole range of concentrations studied, the low-affinity glucose transport system was severely inhibited, and the depression of specific growth rate in *S. cerevisiae* has been found to be closely associated with the inhibition of glucose uptake (C. A. Viegas & I. Sá-Correia, unpublished).

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**References**


Octanoic acid and yeast plasma membrane ATPase


