

## Decrease of H<sub>2</sub>O<sub>2</sub> Plasma Membrane Permeability during Adaptation to H<sub>2</sub>O<sub>2</sub> in *Saccharomyces cerevisiae*\*

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Contrary to what is widely believed, recent published results show that H<sub>2</sub>O<sub>2</sub> does not freely diffuse across biomembranes. The fast removal of H<sub>2</sub>O<sub>2</sub> by antioxidant enzymes is able to generate a gradient if H<sub>2</sub>O<sub>2</sub> is produced in a different compartment from that containing the enzymes (Antunes, F., and Cadenas, E. (2000) *FEBS Lett.* 475, 121–126). In this work, we extended these studies and tested whether an active regulation of biomembranes permeability characteristics is part of the cell response to oxidative stress. Using *Saccharomyces cerevisiae* as a model, we showed that: (a) H<sub>2</sub>O<sub>2</sub> gradients across the plasma membrane are formed upon exposure to external H<sub>2</sub>O<sub>2</sub>; (b) there is a correlation between the magnitude of the gradients and the resistance to H<sub>2</sub>O<sub>2</sub>; (c) there is not a correlation between the intracellular capacity to remove H<sub>2</sub>O<sub>2</sub> and the resistance to H<sub>2</sub>O<sub>2</sub>; (d) the plasma membrane permeability to H<sub>2</sub>O<sub>2</sub> decreases by a factor of two upon acquisition of resistance to this agent by pre-exposing cells either to nonlethal doses of H<sub>2</sub>O<sub>2</sub> or to cycloheximide, an inhibitor of protein synthesis; and (e) *erg3Δ* and *erg6Δ* mutants, which have impaired ergosterol biosynthesis pathways, show higher plasma membrane permeability to H<sub>2</sub>O<sub>2</sub> and are more sensitive to H<sub>2</sub>O<sub>2</sub>. Altogether, the regulation of the plasma membrane permeability to H<sub>2</sub>O<sub>2</sub> emerged as a new mechanism by which cells respond and adapt to H<sub>2</sub>O<sub>2</sub>. The consequences of the results to cellular redox compartmentalization and to the origin and evolution of the eukaryotic cell are discussed.

In general, the first cellular defense against exogenous toxic agents is the plasma membrane, which limits the influx of these agents into cells. For lipophilic agents that cross the plasma membrane, the cells evolved exporting mechanisms to expel the agent, and acquired drug resistance is often associated with the expression of drug efflux pumps such as the P-glycoprotein and the multidrug resistance protein (1). Concerning H<sub>2</sub>O<sub>2</sub>, the most abundant cellular reactive oxygen species, it is widely believed that this agent crosses biomembranes freely, and so mechanisms that impose an extracellular/intra-

cellular gradient for this species have been ignored so far, being implicitly assumed that such gradient does not occur.

However, in extracellular fluids concentrations up to 100 μM H<sub>2</sub>O<sub>2</sub> have been reported (2), whereas the intracellular H<sub>2</sub>O<sub>2</sub> concentration is estimated in the range 0.01–0.1 μM (3, 4), implying the existence of an outside/inside gradient. Supporting this, we showed that H<sub>2</sub>O<sub>2</sub> does not permeate biomembranes freely in a human cell line and that upon exposure to external H<sub>2</sub>O<sub>2</sub> the intracellular consumption of H<sub>2</sub>O<sub>2</sub> catalyzed by antioxidant enzymes is able to generate a gradient of H<sub>2</sub>O<sub>2</sub> across the plasma membrane, resulting in a lower H<sub>2</sub>O<sub>2</sub> concentration in the intracellular milieu (5). These results were later confirmed in an *Escherichia coli* strain by others (6). Furthermore, if an extracellular/intracellular gradient is not formed, the intracellular enzymes are not able to protect individual cells against H<sub>2</sub>O<sub>2</sub>, and it was shown in an *E. coli* strain that does not show a gradient that the increase in catalase activity only protects high density or colonial *E. coli* and does not protect low density or individual *E. coli* (7). Further supporting the importance of the plasma membrane is the observation that after a nonlethal dose of H<sub>2</sub>O<sub>2</sub>, the levels of mRNA that are more repressed are those corresponding to the enzyme HMG-CoA reductase (8). This is the rate-limiting enzyme of the synthesis of sterols, which are key components of the plasma membrane controlling its fluidity (9) and consequently the permeability of this membrane to a species like H<sub>2</sub>O<sub>2</sub> (10).

The possibility that the plasma membrane protects cells against external H<sub>2</sub>O<sub>2</sub> is important because, in most cases, oxidative stress is expected to occur as a result of cells being exposed to an exogenous source of H<sub>2</sub>O<sub>2</sub> (2) such as that produced from environmental factors (*i.e.* redox-cycling agents and radiation) in unicellular organisms or inflammation and injury responses, which in humans are associated with the etiology of several vascular diseases such as atherosclerosis, diabetes, neuronal disorders, and ischemia reperfusion injury (11). On the other hand, intracellular production of H<sub>2</sub>O<sub>2</sub> is expected to have mainly a regulatory role (12) (*e.g.* regulation of cell proliferation (13)), although in some pathological situations, like aging, high levels of oxidative stress may be expected to be generated endogenously (14).

In this work, we tested the hypothesis that the plasma membrane is a key cellular site protecting cells against external H<sub>2</sub>O<sub>2</sub>. If the hypothesis is correct, then the adaptation to H<sub>2</sub>O<sub>2</sub>, *i.e.* the induction of resistance to a high level (usually lethal) of H<sub>2</sub>O<sub>2</sub> by a preliminary low (adaptive) dose of H<sub>2</sub>O<sub>2</sub>, could involve alterations in the plasma membrane permeability to H<sub>2</sub>O<sub>2</sub>. Therefore, we carried out rigorous kinetic studies on the adaptation to H<sub>2</sub>O<sub>2</sub> in *Saccharomyces cerevisiae* (Sc)<sup>1</sup> cells

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<sup>1</sup> The abbreviation used is: Sc, *S. cerevisiae*.

growing in exponential phase, a well known model for adaptation to oxidative stress (15). We further investigated the susceptibility to  $H_2O_2$  in two mutant Sc strains with impaired ergosterol biosynthesis, which show higher membrane permeability to lipophilic agents. We concluded that the plasma membrane is important in the protection against  $H_2O_2$  and that its properties are subjected to regulation leading to a decrease of the permeability coefficient to  $H_2O_2$  during adaptation to this agent.

#### EXPERIMENTAL PROCEDURES

**Materials**—Sc strains used in this work are Y00000 (wild type, genotype BY4741 *MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*), Y02667 (*erg3Δ*, isogenic to BY4741 with *YLR056w::kanMX4*), Y00568 (*erg6Δ*, isogenic to BY4741 with *YML008c::kanMX4*), and Y04718 (*ctt1Δ*, isogenic to BY4741 with *YGR088w::kanMX4*), and they were obtained from EUROSCARF (Frankfurt, Germany).

Yeast extract, bactopectone, yeast nitrogen base, and agar were from Difco (Detroit, MI). Glucose oxidase (*Aspergillus niger*) and digitonin were from Aldrich. Bovine liver catalase, lyticase (*Arthrobacter luteus*), phenylmethylsulfonyl fluoride, and cytochrome *c* were from Sigma. Hydrogen peroxide was obtained from Merck, pyruvate was from Fluka (Buchs, Switzerland), and NADH was from Roche Applied Science.

**Media and Growth Conditions**—Sc cells were inoculated at an  $A_{600}$  of 0.05 and cultured in synthetic complete medium (6.8% (w/v) yeast nitrogen base, 2% (w/v) glucose, 0.002% (w/v) arginine, 0.002% (w/v) methionine, 0.003% (w/v) tyrosine, 0.003% (w/v) isoleucine, 0.003% (w/v) lysine, 0.005% (w/v) phenylalanine, 0.01% (w/v) glutamic acid, 0.015% (w/v) valine, 0.01% (w/v) aspartic acid, 0.0025% (w/v) adenine, 0.04% (w/v) serine, 0.01% (w/v) leucine, 0.005% (w/v) tryptophan, 0.01% (w/v) histidine, 0.02% (w/v) threonine, and 0.0025% (w/v) uracil) at 30 °C with shaking at 160 rpm. For all experiments, the cells in the exponential phase were harvested at  $A_{600} = 0.5$  ( $1 A_{600} = 2-3 \times 10^7$  cells).

**Cell Permeabilization**—Cell membrane permeabilization was achieved by incubating cells with 0.01% (w/v) digitonin dissolved in dimethyl sulfoxide in 0.1 M potassium phosphate buffer, pH 6.5, for 5 min at 30 °C with shaking. Permeabilization was checked by means of lactate dehydrogenase activity measurement, according to Ref. 16.

**Preparation of Spheroplasts**—Spheroplasts were obtained by treating cells for 30 min at 30 °C with lyticase (40 units/ml) in 0.1 M potassium phosphate buffer, pH 6.5, containing 1 M sorbitol, 6.8% (w/v) yeast nitrogen base, and 2% (w/v) glucose. Spheroplast formation was checked by following the decrease of absorption at 660 nm, according to Ref. 17.

**Exposure to and Measurement of  $H_2O_2$  and Cell Survival**—The cells were exposed to steady state hydrogen peroxide concentrations in synthetic complete medium at 30 °C and with shaking at 160 rpm, using glucose oxidase as described in Ref. 18. In brief, steady state levels of  $H_2O_2$  were obtained by adding an initial amount of  $H_2O_2$  together with some glucose oxidase that, by forming  $H_2O_2$ , compensated for the consumption of  $H_2O_2$  by the cells. The consumption of  $H_2O_2$  in Sc cells shows first order decay kinetics with a rate constant of  $0.059 \text{ min}^{-1} A_{600}^{-1}$  (see "Results"). By balancing the initial additions of  $H_2O_2$  and glucose oxidase, a steady state concentration range of 0.15–1 mM was generated.

To establish the sublethal hydrogen peroxide dose, the cells were exposed to steady state concentrations of hydrogen peroxide (150–400  $\mu\text{M}$ ), up to 90 min at 30 °C and with shaking at 160 rpm. Cell survival was monitored by taking samples at 30-min intervals, diluting them, and plating aliquots on YPD plates (1% (w/v) yeast extract, 2% (w/v) bactopectone, 2% (w/v) glucose with 2% (w/v) agar) and counting colonies after 48–72 h. The sublethal dose was defined as the dose that induced about 10% loss in cell viability when compared with control (150  $\mu\text{M}$  under our conditions).

$H_2O_2$  was measured as  $O_2$  release with an oxygen electrode (Hansatech Instruments Ltd., Norfolk, UK) following the addition of catalase (18). Glucose oxidase activity was measured by following  $O_2$  consumption with the oxygen electrode.

**Enzyme Activities and  $H_2O_2$  Consumption**—Crude extracts were prepared by glass bead lysis as described in Ref. 19. Determinations of total protein were done according to Peterson (20).  $H_2O_2$  catabolism in Sc cells has some differences when compared with higher eukaryotes, namely: two catalases are present, the cytosolic catalase T encoded by the gene *CTT1* and the peroxisomal catalase A encoded by the gene *CTA1* (15); in the mitochondrial intermembrane space a cytochrome *c*

peroxidase, encoded by the gene *CCP1*, uses cytochrome *c* to reduce  $H_2O_2$  (21); and the glutathione peroxidases are phospholipid hydroperoxide glutathione peroxidases (encoded by the genes *GPX1*, *GPX2*, and *GPX3*) and do not have selenium in the active center (22). We focused in catalase and cytochrome *c* peroxidase activities because these are the most important enzymes in the  $H_2O_2$  catabolism for SC cells growing in exponential phase (23).

Cytochrome *c* peroxidase activity was measured in protein extracts by following spectrophotometrically the oxidation of cytochrome *c* at 550 nm ( $\epsilon_{550} = 29 \text{ mM}^{-1} \text{ cm}^{-1}$ ) at 25 °C for 4 min by adding 50  $\mu\text{M}$   $H_2O_2$  (24). Ferrocytochrome *c* was prepared as described in Ref. 24. One unit is defined as the quantity of enzyme that catalyzes the oxidation of 1  $\mu\text{mol}$  of cytochrome *c*/min at 25 °C and pH 7.4. Catalase activity in protein extracts was measured spectrophotometrically by following  $H_2O_2$  consumption (initial concentration, 10 mM) at 240 nm at 25 °C for 2 min according to Ref. 25.

The assays performed to measure  $H_2O_2$  consumption rate, either overall or only catalase-driven, were as follows: (a) For catalase activity *in situ*,  $H_2O_2$  consumption (initial concentration, 100  $\mu\text{M}$ ) by permeabilized cells suspended in the permeabilization buffer at 30 °C was followed using the oxygen electrode. This assay can also be considered a measurement of the overall  $H_2O_2$  consumption rate in permeabilized cells because catalase is the only active enzyme (see below). (b) For catalase activity in intact cells, the activity should be called apparent because it is partially limited by the plasma membrane, being thus lower than the real activity (see "Results"). A similar approach to the first assay was used with the following alterations: no digitonin was added to the buffer, and the cells were incubated with 150  $\mu\text{M}$   $H_2O_2$  for 15 min before starting the measurements of  $H_2O_2$  consumption, to oxidize internal pools of reducing equivalents. (c) For overall  $H_2O_2$  consumption rate in intact cells, the rate was measured as in the second assay, but with cells suspended in synthetic complete medium (instead of buffer), and measurements were started immediately after adding 100  $\mu\text{M}$   $H_2O_2$ .

Because in the *ctt1Δ* strain no activity was detected with either the first or second approach but an  $H_2O_2$  consumption rate was observed with the third assay, the first and second assays measured only cytosolic catalase activity, whereas in the third assay other enzymes were also active. Therefore, under our experimental conditions peroxisomal catalase did not represent an important  $H_2O_2$  removing activity. The reason why enzymes other than catalase were not active in the first and second assays is probably the rapid depletion of internal pools of reducing equivalents necessary for these enzymes because of either the plasma membrane permeabilization (in the first assay) or the exposure to a 15-min preoxidation period with  $H_2O_2$  (in the second assay). After being oxidized, they cannot be reduced back because of the lack of carbon sources in the buffer. In the third assay, the pools of reducing equivalents can be maintained in a *quasi* steady state, because incubation is done with intact cells in the presence of carbon sources.

In all cases,  $H_2O_2$  concentrations were plotted semi-logarithmically against time, and catalase activity (or  $H_2O_2$  consumption) was calculated as the slope of the linear fitting (*i.e.* as a first order rate constant).

**Determination of Cellular  $H_2O_2$  Gradient**—The gradient generated by catalase was determined using the principle of enzyme latency (see "Results"). The ratio between apparent catalase activity in intact cells and catalase activity in permeabilized cells was used.

**Statistical Analysis**—The results presented are the means  $\pm$  S.D. of independent experiments. Data statistical analysis was undertaken using either a two-tailed Student *t* test for comparison between means of two different groups or by using analysis of variance and the Tukey-Kramer multiple comparisons test for comparison of more than two different groups.

#### RESULTS

**Adaptation to  $H_2O_2$  Does Not Increase  $H_2O_2$  Removal**—Fig. 1A shows that a 150  $\mu\text{M}$  steady state  $H_2O_2$  concentration triggered an adaptation in Sc cells. In accordance with the data in the literature (23), during this adaptation there were approximate 2- and 3-fold increases in two antioxidant enzymes activities, catalase and cytochrome *c* peroxidase, respectively (Table I). These enzymes are accountable for the removal of  $H_2O_2$  in the cell (23), and so it could be expected that their induction increases the capacity of Sc cells to remove  $H_2O_2$ . Surprisingly, this was not observed (Fig. 1B), and the pseudo-first order rate constant describing the consumption of  $H_2O_2$  by Sc was similar in control and adapted cells (Table I). Therefore, contrary to

FIG. 1. **Adaptation of Sc cells to H<sub>2</sub>O<sub>2</sub>.** A, survival fractions are shown for cells that were exposed to a H<sub>2</sub>O<sub>2</sub> steady state of 0.7 mM for the indicated times. cells were pre-exposed to a H<sub>2</sub>O<sub>2</sub> steady state of 150  $\mu$ M for 90 min (■); cells were pre-exposed to cycloheximide (15  $\mu$ g/ml) during 90 min, a condition that blocks protein synthesis in Sc (43) (▲); whereas control cells were not pre-exposed to neither drug (◆). The averages  $\pm$  S.D. of three to five independent experiments are shown. \*,  $p < 0.01$ ; \*\*,  $p < 0.001$ . B, consumption of H<sub>2</sub>O<sub>2</sub> was followed in control (◆) and in cells pre-exposed to a H<sub>2</sub>O<sub>2</sub> steady state of 150  $\mu$ M for 90 min (■) after adding an initial H<sub>2</sub>O<sub>2</sub> dose of 100  $\mu$ M; the first order kinetic rate constant for the consumption of H<sub>2</sub>O<sub>2</sub> is given by the slope of semi-logarithmic plot (open symbols).

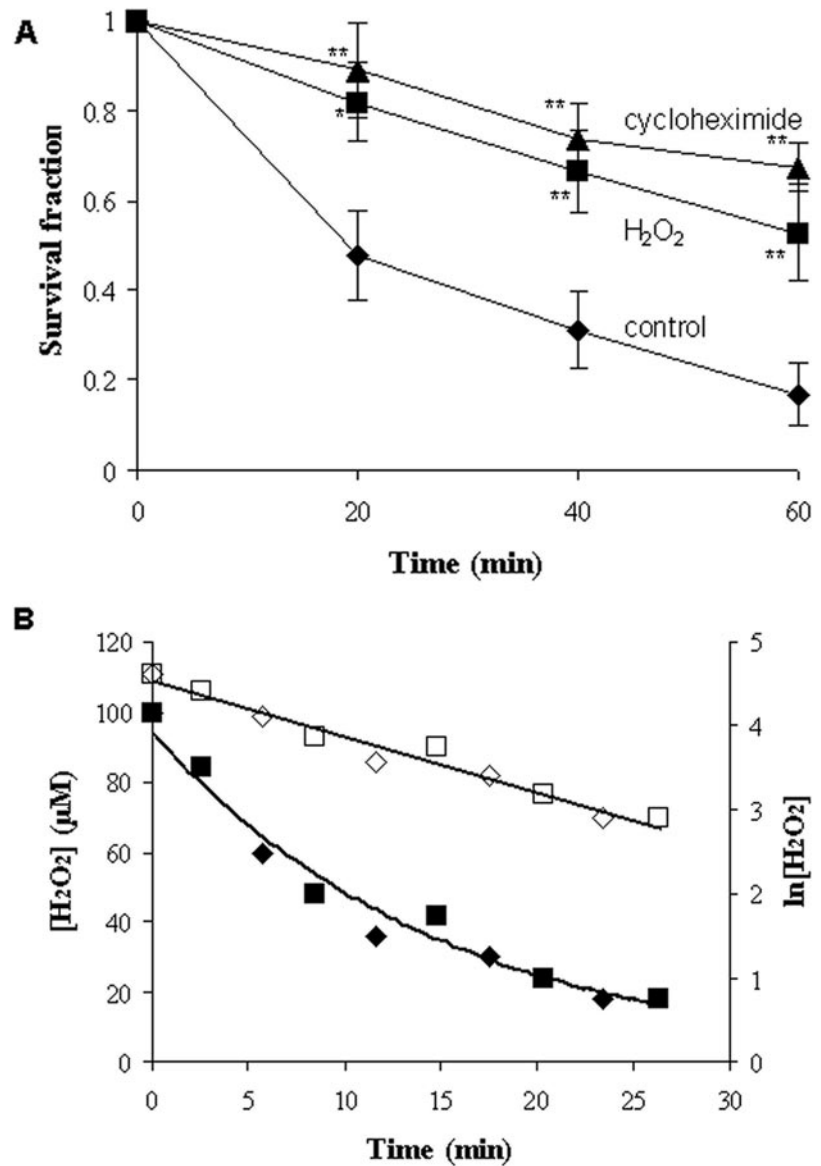


TABLE I

Upon adaptation of Sc cells to H<sub>2</sub>O<sub>2</sub>, intracellular H<sub>2</sub>O<sub>2</sub> removal enzyme activities increase, but the overall H<sub>2</sub>O<sub>2</sub> consumption rate in intact living cells remains constant

Catalase and cytochrome *c* peroxidase activities in cell extracts and the kinetics of H<sub>2</sub>O<sub>2</sub> removal in living cells were measured in control and in Sc cells pre-exposed to a H<sub>2</sub>O<sub>2</sub> steady state of 150  $\mu$ M for 90 min.

	Control cells	Adapted cells
Catalase activity in protein extracts (min <sup>-1</sup> mg <sup>-1</sup> ml)	0.66 $\pm$ 0.14	1.40 $\pm$ 0.09 <sup>a</sup>
Cytochrome <i>c</i> peroxidase activity (milliunits mg <sup>-1</sup> )	0.64 $\pm$ 0.08	2.00 $\pm$ 0.17 <sup>a</sup>
H <sub>2</sub> O <sub>2</sub> consumption rate constant in intact cells (min <sup>-1</sup> A <sub>600</sub> <sup>-1</sup> )	0.059 $\pm$ 0.009	0.062 $\pm$ 0.012

<sup>a</sup>  $p < 0.0001$ ;  $n = 6$ .

what is widely believed, an increased removal of external H<sub>2</sub>O<sub>2</sub> is not part of the mechanism leading to resistance to external H<sub>2</sub>O<sub>2</sub> in Sc.

**H<sub>2</sub>O<sub>2</sub> Does Not Diffuse Freely into Sc Cells**—One possible explanation for the lack of increase in H<sub>2</sub>O<sub>2</sub> consumption rate constant by Sc H<sub>2</sub>O<sub>2</sub>-adapted cells is that H<sub>2</sub>O<sub>2</sub> does not freely diffuse into Sc cells, because of a permeability barrier either at the level of the plasma membrane or the cell wall. If this is the case, then the overall H<sub>2</sub>O<sub>2</sub> consumption rate in Sc cells is not

only determined by the intracellular capacity to remove H<sub>2</sub>O<sub>2</sub>, but it is also dependent on the permeability of Sc cells to H<sub>2</sub>O<sub>2</sub>.

To test the hypothesis that the diffusion of H<sub>2</sub>O<sub>2</sub> into Sc cells limits the overall H<sub>2</sub>O<sub>2</sub> consumption rate, the role of both the cell wall and the plasma membrane as barriers to the diffusion of H<sub>2</sub>O<sub>2</sub> were studied. Firstly, spheroplasts (*i.e.* cells with removed cell wall) from H<sub>2</sub>O<sub>2</sub>-adapted and control cells were obtained. Despite the increased catalase and cytochrome *c* peroxidase activities in spheroplasts produced from adapted cells, the H<sub>2</sub>O<sub>2</sub> consumption rate consumption was similar in spheroplasts produced from control and from adapted cells (not shown). Therefore, the cell wall does not limit H<sub>2</sub>O<sub>2</sub> diffusion into Sc cells, as could be expected because the cell wall has pores that are permeable to low molecular weight molecules (26). Our observation is also consistent with the fact that Sc cell susceptibility to H<sub>2</sub>O<sub>2</sub> is not altered by deleting genes *ECM25*, *ECM33*, and *YOR275c*, which are involved in cell wall integrity (27).

Next, the hypothetical role of the plasma membrane in limiting the diffusion of H<sub>2</sub>O<sub>2</sub> was analyzed by selective permeabilization of the plasma membrane with a low dose of digitonin. The H<sub>2</sub>O<sub>2</sub> consumption rate constant in digitonin-permeabilized adapted cells (0.097  $\pm$  0.009 min<sup>-1</sup> A<sub>600</sub><sup>-1</sup>,  $n = 5$ ) was  $\sim$ 2-fold higher than in permeabilized control cells



( $0.048 \pm 0.004 \text{ min}^{-1} A_{600}^{-1}$ ,  $n = 8$ ). Therefore, it can be concluded that in fact the diffusion of H<sub>2</sub>O<sub>2</sub> across the plasma membrane limits, at least partially, the overall H<sub>2</sub>O<sub>2</sub> consumption rate in Sc cells.

**H<sub>2</sub>O<sub>2</sub> Gradients Are Higher in H<sub>2</sub>O<sub>2</sub>-adapted Sc than in Control Sc Cells**—According to the principle of enzyme latency, a direct consequence of the limited diffusion of H<sub>2</sub>O<sub>2</sub> across the plasma membrane is the formation of gradients between the extracellular and the intracellular milieu when cells are exposed to external H<sub>2</sub>O<sub>2</sub> (5), i.e. the intracellular concentration is lower than the extracellular concentration. This principle states that an enzyme entrapped in a compartment shows a lower activity than when it is free in solution because of the permeability barrier made up by the compartment that limits the diffusion of the substrate to the enzyme (28), and from it Equation 1 is derived (28, 29),

$$\frac{[\text{H}_2\text{O}_2]_{\text{in}}}{[\text{H}_2\text{O}_2]_{\text{out}}} = \frac{k_{\text{perm}}}{k_{\text{perm}} + k_{\text{catabolism}}} = R \quad (\text{Eq. 1})$$

in which  $k_{\text{perm}}$  and  $k_{\text{catabolism}}$  refer to the first order rate constants for the permeation of H<sub>2</sub>O<sub>2</sub> across the plasma membrane and to the intracellular catabolism of H<sub>2</sub>O<sub>2</sub>, respectively, and  $R$  refers to the ratio between the overall H<sub>2</sub>O<sub>2</sub> consumption rate constant in intact cells over the consumption rate constant in permeabilized cells.

Despite its simplicity, three important biological implications can be obtained from the analysis of Equation 1: (a) If  $k_{\text{perm}} \gg k_{\text{catabolism}}$ , then  $[\text{H}_2\text{O}_2]_{\text{in}} = [\text{H}_2\text{O}_2]_{\text{out}}$ , i.e. there is no gradient; but if  $k_{\text{perm}}$  is limiting the consumption of H<sub>2</sub>O<sub>2</sub> (either partially or totally), as observed in Sc cells, a H<sub>2</sub>O<sub>2</sub> gradient is formed. (b) If  $k_{\text{catabolism}}$  increases, the gradient is also increased; so we can expect that in H<sub>2</sub>O<sub>2</sub>-adapted Sc cells, which showed an increase in the activities of catalase and cytochrome *c* peroxidase (Table I), there is a gradient that is larger than in control cells. 3) The determination of this gradient can be based on the experimental measurement of  $R$  (5), which is very helpful because a direct measurement of H<sub>2</sub>O<sub>2</sub> inside the cells is not available.

To determine  $R$ , the overall H<sub>2</sub>O<sub>2</sub> consumption rate constants both in intact cells and in permeabilized cells have to be measured. Experimentally, it was difficult to measure the overall rate of consumption of H<sub>2</sub>O<sub>2</sub> in disrupted cells because after a short period where a rapid and changing consumption rate was observed, the rate of consumption decreased and then remained constant (not shown). Catalase is responsible for this constant rate because this consumption was not observed in the *ctt1Δ* mutant (not shown). Therefore, only the gradient generated by the action of catalase could be measured accurately. In this case, Equation 1 is transformed in Equation 2,

$$\frac{[\text{H}_2\text{O}_2]_{\text{in}}}{[\text{H}_2\text{O}_2]_{\text{out}}} = \frac{k_{\text{perm}}}{k_{\text{perm}} + k_{\text{catalase}}} = R_{\text{catalase}} \quad (\text{Eq. 2})$$

where  $k_{\text{catalase}}$  refers to the first order rate constant describing the intracellular consumption of H<sub>2</sub>O<sub>2</sub> by catalase (i.e. catalase activity) and  $R_{\text{catalase}}$  refers to the ratio between the apparent activity of catalase in intact cells and the activity in permeabilized cells. Because  $k_{\text{catalase}} < k_{\text{catabolism}}$ , the gradient caused by catalase is lower than the gradient when all antioxidant enzymes removing H<sub>2</sub>O<sub>2</sub> are active, but it is a good indication for relative values of the overall gradient when comparing different cells.

As can be seen in Table II, the gradient is significantly higher in H<sub>2</sub>O<sub>2</sub>-adapted Sc cells than in control cells. Therefore, when exposed to the same external H<sub>2</sub>O<sub>2</sub> concentration, adapted cells will endure a lower intracellular H<sub>2</sub>O<sub>2</sub> concentration than control cells. Thus, the increase in catalase and

TABLE II  
The H<sub>2</sub>O<sub>2</sub> gradient across the plasma membrane and the permeability constant are changed upon adaptation to H<sub>2</sub>O<sub>2</sub> in Sc cells  
Adaptation to H<sub>2</sub>O<sub>2</sub> is as in Table I ( $5 \leq n \leq 9$ ).

	Control cells	Adapted cells
Apparent catalase activity in intact cells ( $\text{min}^{-1} A_{600}^{-1}$ )	$0.030 \pm 0.003$	$0.033 \pm 0.005$
Catalase activity in permeabilized cells ( $\text{min}^{-1} A_{600}^{-1}$ )	$0.048 \pm 0.004$	$0.097 \pm 0.009^a$
$R_{\text{cat}}^b$	$0.64 \pm 0.09$	$0.34 \pm 0.06^c$
H <sub>2</sub> O <sub>2</sub> -permeability ( $k_{\text{perm}}^d$ ) ( $\text{min}^{-1} A_{600}^{-1}$ )	$0.083 \pm 0.028$	$0.049 \pm 0.012^c$

<sup>a</sup>  $p < 0.0001$ .

<sup>b</sup>  $R_{\text{cat}}$ , the gradient established by the activity of catalase. It is calculated as the ratio between catalase activity in intact and in permeabilized cells.

<sup>c</sup>  $p < 0.05$ .

<sup>d</sup>  $k_{\text{perm}}$ , the permeability constant of Sc to H<sub>2</sub>O<sub>2</sub> is calculated from a simple algebraic manipulation of Equation 2.

cytochrome *c* peroxidase activities does not cause a higher rate of H<sub>2</sub>O<sub>2</sub> disposal but the formation of a steeper gradient of H<sub>2</sub>O<sub>2</sub> across the plasma membrane.

**H<sub>2</sub>O<sub>2</sub> Adaptation Decreases Plasma Membrane Permeability to H<sub>2</sub>O<sub>2</sub> in Sc Cells**—Having established the rate-limiting role of the plasma membrane for the overall H<sub>2</sub>O<sub>2</sub> consumption and the formation of H<sub>2</sub>O<sub>2</sub> gradients across this membrane, we next investigated whether the permeability to H<sub>2</sub>O<sub>2</sub> is changed during adaptation. If the existence of this permeability barrier is important to provide protection against H<sub>2</sub>O<sub>2</sub>, it could be expected that during H<sub>2</sub>O<sub>2</sub> adaptation changes in the plasma membrane occur to decrease H<sub>2</sub>O<sub>2</sub> diffusion into the cell. To test this hypothesis we used Equation 2 to calculate  $k_{\text{perm}}$  in control and H<sub>2</sub>O<sub>2</sub>-adapted cells (Table II).  $k_{\text{perm}}$  in H<sub>2</sub>O<sub>2</sub>-adapted cells decreased ~2-fold when compared with control cells, indicating that H<sub>2</sub>O<sub>2</sub>-adapted cells are less permeable to H<sub>2</sub>O<sub>2</sub>.

Therefore, in addition to the well known increase in antioxidant enzyme activities during H<sub>2</sub>O<sub>2</sub> adaptation, we showed for the first time that in Sc cells, plasma membrane properties are altered, making it less permeable to H<sub>2</sub>O<sub>2</sub> and thus protecting Sc cells against H<sub>2</sub>O<sub>2</sub>. Two important biological consequences of the combined effect of increased catalase and cytochrome *c* peroxidase activities and decreased plasma membrane permeability to H<sub>2</sub>O<sub>2</sub> are: (a) a significant increase in the gradient between extracellular and intracellular H<sub>2</sub>O<sub>2</sub> during adaptation to H<sub>2</sub>O<sub>2</sub>, because  $k_{\text{catabolism}}$  is increased and  $k_{\text{perm}}$  is decreased in Equation 1 and (b) unaltered H<sub>2</sub>O<sub>2</sub> overall consumption rate in adapted cells, because the increased intracellular capacity for H<sub>2</sub>O<sub>2</sub> removal is compensated by the decreased diffusion of H<sub>2</sub>O<sub>2</sub> into the cell, as observed experimentally (Table I).

**Cycloheximide Causes H<sub>2</sub>O<sub>2</sub> Adaptation and Decreases Plasma Membrane Permeability to H<sub>2</sub>O<sub>2</sub> in Sc Cells**—If the decrease in the plasma membrane permeability to H<sub>2</sub>O<sub>2</sub> is an important regulatory response during adaptation to H<sub>2</sub>O<sub>2</sub>, then other agents that induce resistance to H<sub>2</sub>O<sub>2</sub> could also decrease cell permeability to H<sub>2</sub>O<sub>2</sub>. Cycloheximide, a protein synthesis inhibitor, increases Sc cells resistance to H<sub>2</sub>O<sub>2</sub> (30). The molecular mechanism involved is unknown, and the observation has been difficult to interpret because upon incubation with cycloheximide, catalase and cytochrome *c* peroxidase, as well as other proteins thought necessary for H<sub>2</sub>O<sub>2</sub> adaptation, are probably not induced because of the inhibition of protein synthesis. Therefore, we tested whether cycloheximide-induced plasma membrane changes could explain the observed adaptive effect to H<sub>2</sub>O<sub>2</sub>. In fact, under conditions where a strong adaptive effect was observed (Fig. 1), plasma membrane permeability to H<sub>2</sub>O<sub>2</sub> also decreased from  $0.083 \pm 0.028 \text{ min}^{-1} A_{600}^{-1}$  to  $0.042 \pm 0.013 \text{ min}^{-1} A_{600}^{-1}$  ( $n \geq 8$ ). This decrease is similar to that observed when cells are exposed to an adaptive

TABLE III  
Alteration in ergosterol composition of the plasma membrane in *Sc* cells changes the permeability to H<sub>2</sub>O<sub>2</sub>

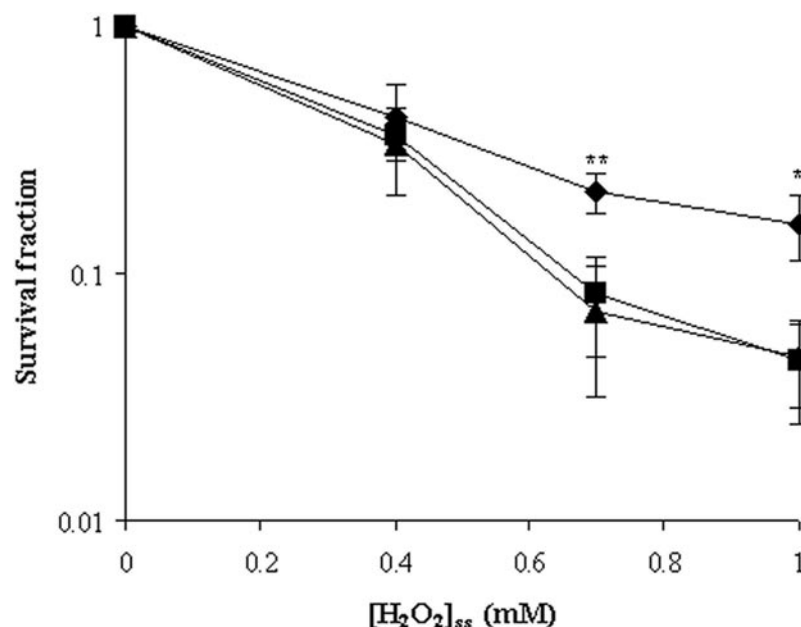
	Wild type	<i>erg3Δ</i>	<i>erg6Δ</i>
H <sub>2</sub> O <sub>2</sub> consumption rate constant in intact cells (min <sup>-1</sup> A <sub>600</sub> <sup>-1</sup> )	0.059 ± 0.009	0.078 ± 0.007 <sup>a</sup>	0.118 ± 0.013 <sup>b</sup>
Cytochrome <i>c</i> peroxidase activity (milliunits mg <sup>-1</sup> )	0.64 ± 0.08	0.68 ± 0.10	0.65 ± 0.15
Catalase activity in permeabilized cells (min <sup>-1</sup> A <sub>600</sub> <sup>-1</sup> )	0.048 ± 0.004	0.026 ± 0.004 <sup>b</sup>	0.048 ± 0.006
Apparent catalase activity in intact cells (min <sup>-1</sup> A <sub>600</sub> <sup>-1</sup> )	0.030 ± 0.003	0.028 ± 0.002	0.050 ± 0.007 <sup>b</sup>
$R_{cat}^c$	0.64 ± 0.09 <sup>b</sup>	1.08 ± 0.19	1.04 ± 0.20

<sup>a</sup>  $p < 0.05$  versus wild type.

<sup>b</sup>  $p < 0.001$  versus the other two strains ( $4 \leq n \leq 9$ ).

<sup>c</sup>  $R_{cat}^c$  calculated as in Table II.

FIG. 2. *Sc* mutants in ergosterol biosynthesis are more susceptible to H<sub>2</sub>O<sub>2</sub> than the wild type strain. Survival fractions are shown for cells that were exposed to a H<sub>2</sub>O<sub>2</sub> steady state between 0.4 and 1 mM for 60 min. ♦, wild type strain; ▲, *erg6Δ*; ■, *erg3Δ*. The averages ± S.D. of three to five independent experiments are shown. \*,  $p < 0.01$ ; \*\*,  $p < 0.001$ .



dose of H<sub>2</sub>O<sub>2</sub>, and because cycloheximide is not an oxidant, this result is particularly relevant to support the possibility that the regulation of the plasma membrane permeability is a general mechanism by which cells acquire resistance to H<sub>2</sub>O<sub>2</sub>.

**Changes in Ergosterol Composition Make *Sc* Cells More Susceptible to H<sub>2</sub>O<sub>2</sub>**—To further test the importance of the plasma membrane in the protection against H<sub>2</sub>O<sub>2</sub>, we compared the susceptibility to H<sub>2</sub>O<sub>2</sub> of two *Sc* strains, which have defective membranes, with that of the wild type *Sc* strain. The two strains used (*erg3Δ* and *erg6Δ*) have a defect in the pathway of ergosterol biosynthesis: *erg3Δ* mutants lack a C-5 desaturase producing ergosta-7,22-dienol instead of ergosterol (31), and *erg6Δ* mutants lack a C-24 methyltransferase producing zymosterol and colessta-5,7,24-trienol instead of ergosterol (32). In both mutants the membrane biophysical properties are changed, resulting in increased permeability to lipophilic compounds (9). As can be observed in Table III, these mutants show a higher H<sub>2</sub>O<sub>2</sub> consumption rate constant when compared with wild type cells, despite a similar cytochrome *c* peroxidase activity and similar or lower catalase activity in permeabilized cells. This can be interpreted by increased membrane permeability to H<sub>2</sub>O<sub>2</sub>. To further confirm this, the H<sub>2</sub>O<sub>2</sub> gradient in these mutants was calculated by a similar approach to that used for wild type cells. As shown in Table III, in *erg3Δ* and *erg6Δ* cells catalase activities both in intact and in permeabilized cells are identical, *i.e.* catalase-driven gradients are not produced; thus, the plasma membrane permeability to H<sub>2</sub>O<sub>2</sub> is increased in these mutants.  $k_{perm}$  could not be calculated because in the absence of a gradient the only information that could be obtained was that  $k_{perm} \gg k_{catalase}$ .

If the gradients are in fact important, then it could be expected that both mutants have a similar susceptibility to H<sub>2</sub>O<sub>2</sub>

and that this susceptibility is higher than that shown by the wild type strain. As can be seen in Fig. 2, this was the observed behavior when cells were subjected to H<sub>2</sub>O<sub>2</sub> steady state incubations. This confirms the role of the plasma membrane for the protection against H<sub>2</sub>O<sub>2</sub>.

## DISCUSSION

Fundamental biochemical aspects regarding cellular protection against H<sub>2</sub>O<sub>2</sub> were made clear in this work and changed our view on the role of the plasma membrane as a protection barrier against H<sub>2</sub>O<sub>2</sub>. Contrary to the commonly accepted concept that H<sub>2</sub>O<sub>2</sub> diffuses freely across biomembranes, we showed that this is not the case in *Sc* cells and that as consequence gradients are formed across the plasma membrane when *Sc* cells are exposed to exogenous H<sub>2</sub>O<sub>2</sub>. Most importantly, the role of the plasma membrane in the generation of gradients, by constituting a barrier against the diffusion of H<sub>2</sub>O<sub>2</sub>, is not a passive one because its permeability to H<sub>2</sub>O<sub>2</sub> is subjected to regulation, as shown by our studies of adaptation to H<sub>2</sub>O<sub>2</sub>. In fact, upon acquisition of H<sub>2</sub>O<sub>2</sub> resistance, by exposure either to a nonlethal adaptive dose of H<sub>2</sub>O<sub>2</sub> or to cycloheximide, permeability toward H<sub>2</sub>O<sub>2</sub> is decreased, increasing the plasma membrane protective role against H<sub>2</sub>O<sub>2</sub>.

Concerning the detailed molecular mechanisms that are responsible for the change in the plasma membrane permeability properties, they were not addressed in this work. The plasma membrane of *Sc* cells is a complex biological site, and a multitude of factors can change its permeability properties. Four observations, however, point to a repression of the ergosterol biosynthesis pathway as a possible mechanism: (a) for a nonlethal dose of H<sub>2</sub>O<sub>2</sub>, the levels of mRNA that are more repressed are those corresponding to the enzyme HMG-CoA reduc-

tase (8), the rate-limiting enzyme in the ergosterol biosynthesis pathway; (b) H<sub>2</sub>O<sub>2</sub> inhibits the enzyme HMG-CoA reductase (33); (c) cycloheximide, a protein synthesis inhibitor that can possibly lead to a lower steady state level of HMG-CoA reductase, decreases plasma membrane permeability to H<sub>2</sub>O<sub>2</sub> (this work); and (d) *erg3Δ* and *erg6Δ*, which accumulate altered sterols (9) and have the ergosterol biosynthesis pathway up-regulated (31, 34), show increased permeability to H<sub>2</sub>O<sub>2</sub> (this work).

Our results conciliate the contradictory findings in the literature concerning whether catalase is important in the adaptive response of cells at low densities; it was shown in an *E. coli* strain that an increase in catalase activity does not protect low density or individual *E. coli* (7), which was attributed to the inability of this strain to form an extracellular/intracellular H<sub>2</sub>O<sub>2</sub> gradient; however, in *Sc* cells catalase was found to be important even at low density conditions, a discrepancy that was not explained (23). If the gradient concept is taken into account this behavior is expected because the imposition of a gradient is much more difficult in *E. coli* than in *Sc* cells because the surface to volume ratio is much higher in the relative smaller *E. coli* cells than in the relative larger *Sc* cells. The H<sub>2</sub>O<sub>2</sub> gradient in *Sc* cells found in the present work supports this explanation.

Concerning biomembranes other than the plasma membrane, it is expectable that they also constitute permeability barriers to H<sub>2</sub>O<sub>2</sub>, and in fact we have estimated a gradient of 3 across the peroxisomal membrane in Jurkat T-cells (5). The concept of intracellular gradients of H<sub>2</sub>O<sub>2</sub> has important implications for cellular redox compartmentalization (35). For example, it helps to understand how it is possible to achieve simultaneously oxidative conditions in cytosol and reductive conditions in the nucleus, which are necessary for activation of NF-κB by H<sub>2</sub>O<sub>2</sub> (36, 37), or how a mild oxidative environment in the endoplasmic reticulum necessary for a correct protein folding (38) can be maintained in a overall reductive cellular environment.

In addition to the well recognized pathological relevance of extracellular H<sub>2</sub>O<sub>2</sub> in inflammation and injury responses (11), another situation in which extracellular H<sub>2</sub>O<sub>2</sub> was also a potential major hazard to the cells, constituting a possible evolutionary pressure for the origin of the eukaryotic cell, was during the pre-Cambrian Earth. Approximately between 2 and 3 billion years ago two events occurred: (a) the slow conversion of a small prokaryote ancestor into a large phagocytic cell possessing most of the characteristics of the modern eukaryotes and able to acquire endosymbionts (39, 40) and (b) massive generation of H<sub>2</sub>O<sub>2</sub> resulting from the oxidation of the abundant Fe<sup>2+</sup> ions to Fe<sup>3+</sup> by the O<sub>2</sub> produced by cyanobacteria, which lead to the formation of ferric oxide deposits (41). H<sub>2</sub>O<sub>2</sub> concentrations in the aqueous environment during this period have been estimated in the millimolar range (7). Taking into consideration that during this period cellular iron-chelating systems would not be yet fully optimized, H<sub>2</sub>O<sub>2</sub> would be extremely hazardous to cells, because upon diffusion into the cell it would easily react with Fe<sup>2+</sup> producing hydroxyl radicals. Supporting this view, catalase has been described as an ancient enzyme, and many of the primitive cells would already have this enzyme (42). However, in the absence of a gradient, catalase does not protect individual cells (7), and therefore smaller cells, where the gradient is either nonexistent or smaller, would be more susceptible to H<sub>2</sub>O<sub>2</sub>. The driving forces for the increase in the cell volume during the slow conversion of prokaryotes to eukaryotes remain largely unknown, and we speculate, that the protective effect of the formation of a steeper H<sub>2</sub>O<sub>2</sub> gradient in larger cells may have been a driving force for

the increase in the cell volume and consequently the appearance of eukaryotic cells.

In conclusion, there are now several studies, performed in a range of organisms, *E. coli* (6), *Sc* cells (this work), and human cell lines (Jurkat T-cells (5); MCF-7),<sup>2</sup> that clearly show that H<sub>2</sub>O<sub>2</sub> does not diffuse freely across plasma membranes. Furthermore, we showed here for the first time that the plasma membrane is not a passive actor in the defense against H<sub>2</sub>O<sub>2</sub> but is subjected to regulation to fulfill its protective role. Therefore, more attention should be paid to the role of biomembranes when studying the biological actions of H<sub>2</sub>O<sub>2</sub>, and the common assumption that H<sub>2</sub>O<sub>2</sub> diffuses freely across biomembranes should be avoided.

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<sup>2</sup> V. Oliveira-Marques, H. S. Marinho, L. Cyrne, and F. Antunes, unpublished observations.

**Decrease of H<sub>2</sub>O<sub>2</sub> Plasma Membrane Permeability during Adaptation to H<sub>2</sub>O<sub>2</sub> in  
*Saccharomyces cerevisiae***

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