

Electron transport chain of *Saccharomyces cerevisiae* mitochondria is inhibited by H₂O₂ at succinate-cytochrome c oxidoreductase level without lipid peroxidation involvement

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Abstract

The deleterious effects of H₂O₂ on the electron transport chain of yeast mitochondria and on mitochondrial lipid peroxidation were evaluated. Exposure to H₂O₂ resulted in inhibition of the oxygen consumption in the uncoupled and phosphorylating states to 69% and 65%, respectively. The effect of H₂O₂ on the respiratory rate was associated with an inhibition of succinate-ubiquinone and succinate-DCIP oxidoreductase activities. Inhibitory effect of H₂O₂ on respiratory complexes was almost completely recovered by β-mercaptoethanol treatment. H₂O₂ treatment resulted in full resistance to Q_O site inhibitor myxothiazol and thus it is suggested that the quinol oxidase site (Q_O) of complex III is the target for H₂O₂. H₂O₂ did not modify basal levels of lipid peroxidation in yeast mitochondria. However, H₂O₂ addition to rat brain and liver mitochondria induced an increase in lipid peroxidation. These results are discussed in terms of the known physiological differences between mammalian and yeast mitochondria.

Keywords: Yeast, mitochondria, oxygen consumption, respiratory chain, H₂O₂, peroxidation

Introduction

In cells, the mitochondrial electron transport chain (ETC) is one of the most important sources of reactive oxygen species (ROS) [1]. ROS generation in ETC may be promoted *in vitro* through addition of certain inhibitors of respiratory complexes, whereas some pathological situations, such as cardiomyopathy [2,3], hyperglycaemia [4], calcium overload [5] or genetic defects in proteins that constitute respiratory complexes [6] may increase the *in vivo* rate of mitochondrial ROS production. Because ROS are short-lived species and react readily with molecules

surrounding their generation site, it should be expected that lipids and proteins from mitochondrial membranes are their main target when mitochondrial ROS generation is augmented. Hence, the study of oxidative stress effects on ETC components has become important, because mitochondrial oxidative stress-related dysfunction could be implicated in a wide range of disorders, including liver damage by hepatitis B [7], heart ischemia-reperfusion [8] and ageing [9,10].

A number of studies have focused on lipid peroxidation as the main factor of ETC inhibition

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by ROS. Exposure of bovine heart submitochondrial particles to ascorbate plus ADP/Fe²⁺ diminished the ubiquinone content and succinate dehydrogenase and cytochrome *c* oxidase activities. These effects were paralleled by an increase in lipid peroxidation [11]. In rat synaptosomes, the activities of complexes II, III and V were partially inhibited by ascorbate-iron treatment. In this system, lipophilic antioxidants protected the activity of complex III, so it was suggested that lipid peroxidation was involved in inhibition. However, protein oxidation could also be involved in the effects on these complexes [12]. In mitochondria isolated from rat hearts subjected to an ischemia-reperfusion cycle, the rate of H₂O₂ production increased when using succinate as respiratory substrate, leading to lipid peroxidation-associated cardiolipin loss and diminution in state 3 respiration rate. This last effect was attributed to a partial inhibition in activities of complexes I and III [13,14].

Despite the above described, some authors have reported that ETC may be oxidatively damaged by mechanisms which do not necessarily involve peroxidative damage to lipids. Giulivi et al. [15] reported the preferential attack of oxygen singlet to proteic components of ETC over the attack to lipidic components of mitochondrial membranes. In beef heart submitochondrial particles exposed to increasing doses of hydroxyl radical (OH[•]) [16], lipophilic antioxidants that inhibit lipid peroxidation did not prevent the oxidative inactivation of NADH and succinate dehydrogenases. Similarly, the incubation of rat brain mitochondria with FeSO₄/ascorbate mixtures (an OH[•] generating system) augmented lipid peroxidation levels and inhibit ETC function. However, the lipid peroxidation inhibitor, butylated hydroxytoluene, did not avoid inhibition in ETC function but did prevent the increase in lipid peroxides and the OH[•]-stimulated carbonylation of proteins [17]. Therefore, these reports suggest that lipid peroxidation-independent oxidative damage might be of critical importance in the mechanism of ETC inactivation.

Even when a more extensive characterization about the effects of OH[•] and superoxide radicals has been done, less attention has been put in the effects of H₂O₂ on ETC functionality. This aspect could be potentially important since H₂O₂ has been considered the most important ROS at cellular level [18], mitochondria are the principal source of H₂O₂ [18] and ETC components of mitochondria are enriched in cytochromes and Fe-S centres, which are important targets of oxidative attack by this ROS [19,20].

A large body of experimental evidence has demonstrated a direct correlation between susceptibility to peroxidation of membrane lipids and its degree of unsaturation [21–24]. In fact, this correlation has

also been shown at mitochondrial level. In rat mitochondria the three main membrane phospholipids (cardiolipin, phosphatidylcholine, phosphatidylethanolamine) are composed of the highly unsaturated docosahexaenoic acid (22:6) and arachidonic acid (20:4) and they are significantly more sensitive to lipid peroxidation, whereas, in membranes from pigeon mitochondria, which are more resistant to lipid peroxidation than rat mitochondria, these highly unsaturated fatty acids are substituted by linoleic acid (18:2) [23,24].

Saccharomyces cerevisiae mitochondria present some important differences in comparison to rat mitochondria, including (a) the lack of a rotenone-sensitive complex I [25,26] and (b) when grown using lactate as a carbon source, the membrane fatty acid composition of mitochondria consists mainly of C16:1 and C18:1 fatty acids [27]. The last feature of yeast mitochondria probably results in higher resistance to oxidative stress. Hence, the way in which ROS inhibit yeast ETC could be different from their action on rat mitochondria, because yeast mitochondria might be less sensitive to lipid peroxidation due to their lipid composition. For the same reason, yeast mitochondria may be a more suitable system than lipophilic antioxidants to discriminate the direct effects of ROS on components of ETC from those attributable to lipid peroxidation.

The toxicity of H₂O₂ on yeast mitochondria was studied. It was found that yeast mitochondria are highly resistant to ROS and, in addition, the mechanism of damage seems to be circumscribed to thiol groups of proteins since thiol reductant β-mercaptoethanol was able to revert the damaging effect of H₂O₂ on dehydrogenase activities of ETC, phospholipids in the membrane were not damaged, cytochrome content was not altered and hydroxyl radical scavenger mannitol or iron quelant EDTA were not able to prevent the inhibitory effect exerted by H₂O₂ on ETC reactions. It was observed that the Q₀ site in complex-III was the most affected function on the ETC.

Materials and methods

Materials

Zymolyase 20T was obtained from ICN Biomedicals, Inc. (Aurora, OH). H₂O₂ (38% v/v), succinic acid, β-mercaptoethanol, 2,6-dichlorophenolindophenol (DCIP), potassium cyanide, tetramethyl-p-phenylenediamine (TMPD), cytochrome *c*, flavone, antimycin A and myxothiazol were obtained from Sigma Chemical. Co. (St. Louis, MO). Stigmatellin was obtained from Fluka Chemie GmbH (Buchs, Switzerland). All other reagents were of the highest purity commercially available.

Biologicals

An industrial wild type diploid strain of *Saccharomyces cerevisiae*, Yeast Foam was used (kind gift from Professor Michel Rigoulet, IBGC, U. of Bordeaux-2, France). Three-month old, male Wistar rats from our local colony were used for isolation of liver and brain mitochondria.

Isolation of yeast mitochondria

Cells of Yeast Foam were grown aerobically at 28°C in a medium containing 0.12% (NH₄)₂SO₄, 0.1% KH₂PO₄, 1% yeast extract, 2% DL-lactate (pH 5.0 with NaOH) and harvested in mid-exponential growth phase (OD₅₅₀ = 3.5–4.0). Mitochondria were isolated from spheroplasts as described previously [28,29], except that zymolase was used instead of cytohelicase.

Isolation of rat brain mitochondria

Rat brain mitochondria were isolated by differential centrifugation in a Percoll gradient as described [30,31], with some modifications. Briefly, rats were decapitated and the brain was extracted and placed in cold medium containing 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 0.5% bovine serum albumin and 10 mM MOPS (pH 7.4). The brain was homogenized manually in a glass homogenizer and centrifuged at 400 g; the supernatant was centrifuged at 9 000 g. Centrifugations were carried out for 10 min at 4°C. The pellet was resuspended in 15% Percoll and placed in a discontinuous Percoll (23% and 40%) gradient. The gradient was centrifuged at 30 700 g, during 6 min, band 3 was extracted, diluted 1:4, centrifuged and washed at 16 700 g in the isolation medium supplemented with 0.5% bovine serum albumin for 10 min. The last centrifugation was done at 6900 g for 10 min.

Isolation of rat liver mitochondria

Rat liver mitochondria were prepared from the same rats used for brain mitochondria isolation. Once the liver was extracted, it was cut into small pieces and homogenized. The homogenate was subjected to differential centrifugation as described previously [32]. In all cases, mitochondrial protein concentration was assayed by Biuret assay [33].

Treatments with H₂O₂

Treatments were performed before each assay at 4°C and mitochondrial protein at a concentration of 0.3 mg/ml. Mitochondria were suspended in 50 mM KH₂PO₄ buffer (pH 7.6 with NaOH), except for oxygen consumption measurements, where mitochondrial protein (0.3 mg/ml) was suspended in a medium with 0.6 M sucrose, 2 mM EGTA, 4 mM

KH₂PO₄, 10 mM Tris-maleate, pH 6.8. This was done because incubation of mitochondria in isotonic KH₂PO₄ buffer inhibited oxygen consumption in intact mitochondria due to swelling. H₂O₂ was added at the beginning of incubations at the concentrations indicated in the legend to each figure. For experiments of protection with EDTA or mannitol, mitochondria were incubated with 50 μM EDTA or 10 mM mannitol during 15 min previous to treatment with H₂O₂. For experiments to determinate reversion of the H₂O₂ effects by β-mercaptoethanol, mitochondria were incubated during 30 min with β-mercaptoethanol after treatments with H₂O₂. To avoid interferences due to cytochromes release in cytochrome spectra experiments and unspecific reduction of DCIP by β-mercaptoethanol during measurements of succinate-DCIP oxidoreductase activity, H₂O₂ was removed to carry out both determinations at the end of the treatment by centrifugating mitochondrial solution at 8700 g during 15 min and mitochondrial pellet were re-suspended in 50 mM KH₂PO₄ buffer (pH 7.6 with NaOH) at adequate concentration and volume for each determination. Controls were treated in the same way except for H₂O₂ addition.

Oxygen consumption

The rate of cyanide-sensitive oxygen consumption in resting state (state 4), phosphorylating state (state 3) and uncoupled state (state U) was monitored at room temperature using an YSI 5300 biological oxygen monitor (Yellow Springs Instrument, Yellow Springs, OH), equipped with a Clark-type electrode. Pre-treated mitochondria (0.3 mg/ml) were placed in a glass chamber with constant stirring, containing 3.0 ml of a medium with 0.6 M sucrose, 2 mM EGTA, 4 mM KH₂PO₄, 10 mM Tris-maleate, pH 6.8. Respiration in states 3 and U was initiated with 300 μM ADP and 2 μM FCCP, respectively.

Succinate dehydrogenase activity

This enzymatic activity was measured at room temperature in permeabilized mitochondria, following the TTFA sensitive secondary reduction of DCIP [34]. Mitochondria were permeabilized with Triton X-100 as reported by Hallberg et al. [35] and treated with H₂O₂ as indicated above. H₂O₂ was removed at the end of the treatment to eliminate unspecific reduction of DCIP by β-mercaptoethanol by centrifugating mitochondrial solution at 8700 g during 15 min and mitochondrial pellet was resuspended at a concentration of 0.3 mg in a final 1 ml volume of reaction mixture contained 50 mM KH₂PO₄ phosphate buffer (pH 7.6), 10 mM sodium succinate (pH 7.6), 50 μM flavone, 1 μg antimycin A and 1 mM KCN. After incubating for 3 min with inhibitors, the reaction was initiated with 80 μM DCIP. Reaction

was stopped by the addition of 1 mM TTFA. Absorbance changes were recorded in a SLM Aminco DW 2000 spectrophotometer, in the dual wavelength mode at 600–590 nm. The activity was calculated from the slope of the absorbance plot using the molar extinction coefficient of $21 \text{ mM}^{-1} \text{ cm}^{-1}$ for DCIP. The TTFA-sensitive activity was calculated by subtracting the activity in the presence of TTFA plus succinate to the activity stimulated only with succinate.

Succinate cytochrome c oxidoreductase activity

The antimycin A sensitive succinate-mediated reduction of cytochrome *c* was followed by measuring the reduction of cytochrome *c* in permeabilized mitochondria by mild detergent treatment [35]. The reaction mixture contained 50 mM KH_2PO_4 (pH 7.6), 1.5 mg cytochrome *c*, 50 μM flavone and 1 mM KCN in a final 1 ml volume. Pre-treated mitochondrial protein (0.1 mg) was added and, after 3 min, the reaction was initiated by adding 10 mM sodium succinate (pH 7.6). The reaction was stopped by the addition of 1 μg antimycin A. Absorbance changes at 550–540 nm were recorded as described above. The rate of cytochrome *c* reduction was calculated from the slopes of the absorbance plots using a molar extinction coefficient of $19.1 \text{ mM}^{-1} \text{ cm}^{-1}$ for cytochrome *c*. The antimycin-sensitive reduction of cytochrome *c* was calculated by subtracting the activity in the presence of antimycin plus succinate to the activity stimulated only with succinate.

Cytochrome oxidase activity

This activity was evaluated by measuring the cyanide-sensitive oxygen consumption at room temperature using an YSI 5300 biological oxygen monitor, equipped with a Clark-type electrode. The reaction mixture contained 50 mM KH_2PO_4 buffer (pH 7.6), 1 μg antimycin A, 50 μM TMPD and 0.9 mg detergent-solubilized mitochondrial protein in a final concentration of 0.3 mg/ml. The assay was started by the addition of 5 mM sodium ascorbate or 0.6 mg oxidized cytochrome *c* (pH 7.6) and stopped by the addition of 100 μM KCN.

Cytochrome spectra

Cytochrome spectra were obtained by measuring at room temperature the reduced minus oxidized spectra with a SLM Aminco DW 2000 spectrophotometer fitted in the split mode. To eliminate the possibility of cytochromes released by H_2O_2 treatment interfering with the results, pre-treated mitochondrial samples (2 mg) were centrifuged at 8700 *g* during 15 min and re-suspended in a medium with 0.6 M sucrose, 10 mM Tris maleate (pH 6.8), 4 mM KH_2PO_4 ,

2 mM EGTA and 1 mM KCN. bc_1 complex inhibitors were added 5 min before spectra recording. Reference and sample cuvettes were treated in the same way and the baseline was recorded. Then a small amount of KFeCN was added to the reference cuvette while 10 mM succinate or, when indicated, a small quantity of sodium dithionite, was added to the sample cuvette. These cuvettes were used to record the differential spectra. Spectra were scanned between 500–580 nm. Cytochrome contents were calculated using the following wavelengths and molar extinction coefficients: cytochrome *c* + *c*₁, $\Delta A = 552 \text{ nm}$ minus 540 nm and $\epsilon = 19.1 \text{ mM}^{-1} \text{ cm}^{-1}$; cytochrome *b*, $\Delta A = 562 \text{ nm}$ minus 575 nm and $\epsilon = 20 \text{ mM}^{-1} \text{ cm}^{-1}$ [11].

Lipid peroxidation measurements

The extent of lipid peroxidation was determined with the thiobarbituric acid (TBA) assay [36]. Mitochondria were treated with H_2O_2 as indicated above and later combined with 2 ml of an acid mixture containing 0.25 N HCl, 15% w/v trichloroacetic acid and 0.375% w/v thiobarbituric acid. This combination was heated for 15 min in a boiling water bath, cooled on ice and centrifuged at 1 000 *g* for 5 min. The absorbance of the supernatant was measured at 535 nm against a blank containing all the reagents minus mitochondrial protein. A Perkin Elmer Lambda 18 UV/vis spectrometer was used. The results were calculated using the molar extinction coefficient for malondialdehyde and expressed in terms of nanomoles of TBA reactants (TBARS) per milligram of protein.

Results

Isolated yeast mitochondria were incubated for 30 min in the presence of increasing concentrations of H_2O_2 and the effects on the rate of oxygen consumption were tested. Measurements were conducted in both the resting state (state 4) and the phosphorylating state (state 3). In state 4 (Figure 1A) the addition of increasing concentrations of H_2O_2 resulted in a progressive inhibition of respiration, reaching a significant decrease in 51% and 60%, with 0.5 mM and 1.0 mM, respectively. A more pronounced effect was mediated by H_2O_2 in state 3 (Figure 1B), resulting in a decrease in respiration from the lowest concentration tested (0.125 mM). Wide variations in the concentration of H_2O_2 (0.25–1.0 mM) exhibited a stronger inhibitory effect (60–75%) (Figure 1B).

Addition of the protonophore FCCP to mitochondria dissipated membrane potential; thus, respiratory chain activity is stimulated (state U respiration) to compensate membrane potential dissipation. In this way, it is possible to study the maximal activity of respiratory chain. On this basis, respiration in the

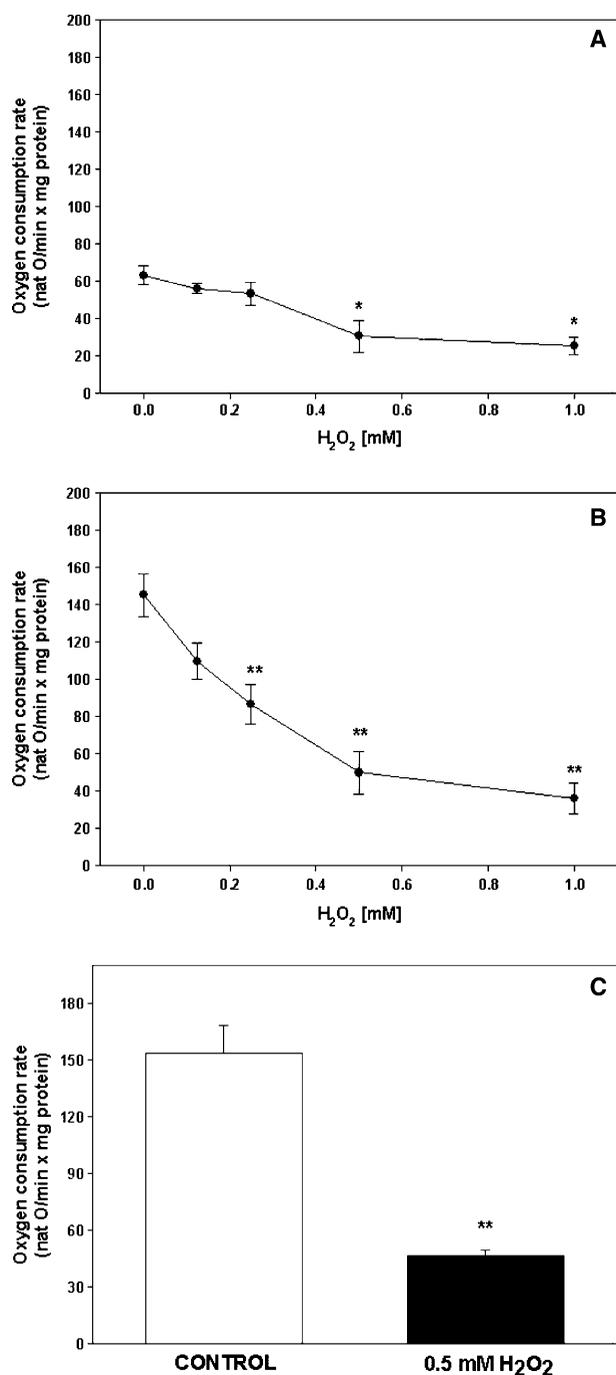


Figure 1. Effect of H₂O₂ on the rate of O₂ uptake in state 4 (A), state 3 (B) or uncoupled state (C). Mitochondria were incubated during 30 min at 4°C in respiration medium (0.6 M sucrose, 2 mM EGTA, 4 mM KH₂PO₄, 10 mM Tris-maleate), with indicated amounts of H₂O₂. After pre-incubation, oxygen uptake was measured as described in Materials and methods. Data are presented as mean \pm SE of >3 independent experiments. Significantly different when compared to control (0 mM H₂O₂) (* p < 0.05, ** p < 0.01).

presence of FCCP was measured to discard that impairment on ATP synthesis was responsible for the inhibition observed in state 3 respiration. In Figure 1C it can be observed that 0.5 mM H₂O₂ inhibited respiration to a similar degree than observed in state 3 at the same concentration of H₂O₂ (65% and 69% in

state 3 and state U, respectively). This result and the partial inhibitory effect on respiration even to the highest concentration of H₂O₂ suggest that damage was exerted at the level of at least one of the respiratory complexes. As well, impairment in ATP synthesis probably is not participating in the observed effects.

Control experiments were carried out to eliminate the possibility that oxygen produced by spontaneous decomposition of H₂O₂ may produce an artificial inhibition on respiration. As Figure 2A shows, oxygen consumption was not detected when this parameter was evaluated in respiratory medium plus 1.0 mM H₂O₂ for 30 min at 4°C. Also, oxygen consumption was not detected in mitochondria treated in the same way and inhibited with 0.1 mM KCN (Figure 2B). Together, these results validate the inhibition on respiration induced by H₂O₂.

Even at the highest concentrations (1.0 mM), H₂O₂ addition resulted in only a partial inhibition, suggesting that electron flow through ETC was compromised at some point. To locate the site of inhibition, the activities of some partial reactions occurring during electron transport in ETC were measured. Succinate-DCIP oxidoreductase activity (complex II) was diminished by 23% and 30% with low (0.125 mM) and intermediate (0.250 mM) concentrations of H₂O₂ (Figure 3A), respectively. Higher quantities of H₂O₂ (0.5–1.0 mM) did not produce an expected inhibitory effect, reaching just 40–45%. A higher sensitivity to H₂O₂ challenge was detected in succinate-cytochrome *c* oxidoreductase activity

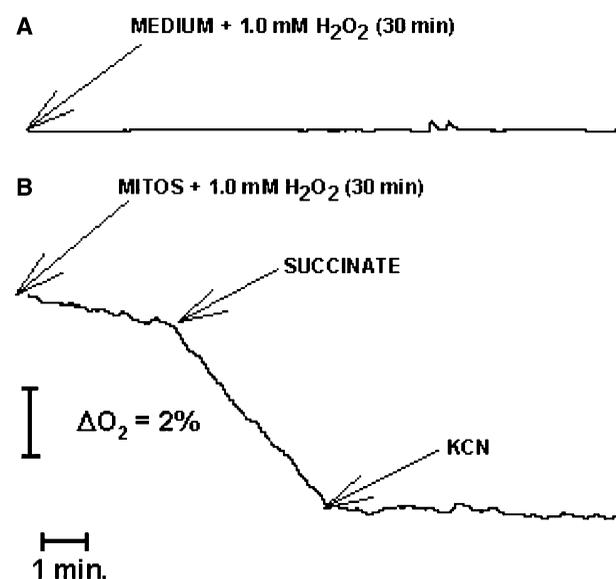


Figure 2. Influence of H₂O₂ decomposition on oxygen consumption determinations. Respiration medium (A) or respiration medium plus 0.9 mg mitochondria (B) were incubated during 30 min at 4°C with 1.0 mM H₂O₂. Then, oxygen production was evaluated with a Clark-type oxygen electrode as described in Materials and methods. (A). Experiment was repeated at least three times. Data are from a representative experiment.

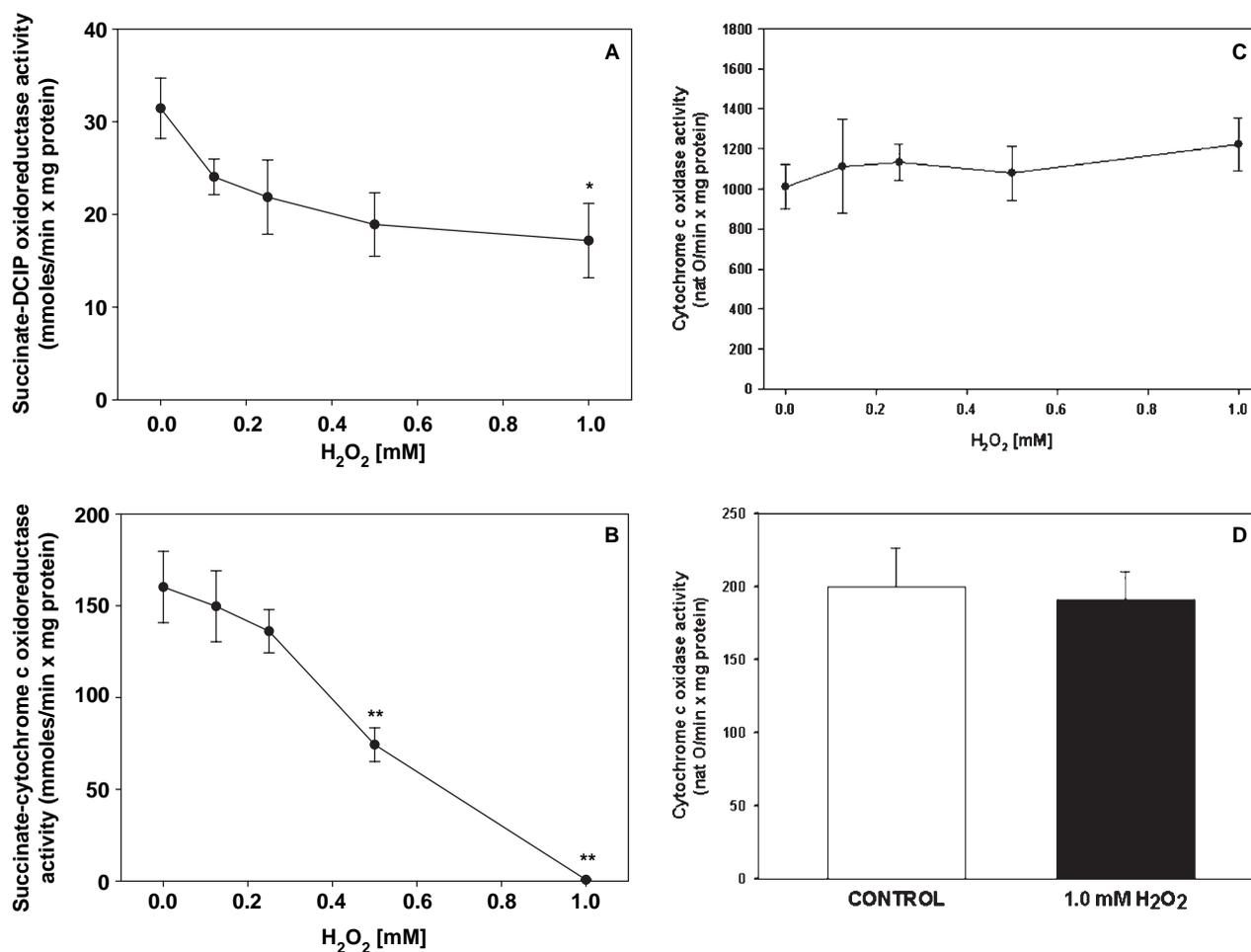


Figure 3. Effect of H₂O₂ on partial reactions of ETC. Triton-solubilized mitochondria were incubated during 30 min at 4°C in 50 mM KH₂PO₄ buffer with indicated concentrations of H₂O₂ under conditions described in Materials and methods. Succinate-DCIP oxidoreductase (A), succinate-cytochrome *c* oxidoreductase (B), cytochrome oxidase (C) or cytochrome *c* oxidase (D) activities were measured as described in Materials and methods. Results are expressed as the mean ± SE of >3 independent experiments. Significantly different when compared to control (0 mM H₂O₂) (**p* < 0.05, ***p* < 0.01).

(complex II–III segment). In this case, a more clear correlation between concentration of H₂O₂ and activity was found; half of the activity was lost when mitochondria was incubated with 0.5 mM, while 1.0 mM H₂O₂ fully inhibited this activity (Figure 3B).

In contrast, cytochrome oxidase activity (complex IV) was completely insensitive to all concentrations of H₂O₂ tested (0.125–1.0 mM), whatever TMPD (Figure 3C) or cytochrome *c* (Figure 3D) were used as electron donor. It must be pointed out that both TMPD and cytochrome *c* bind to cytochrome *c* oxidase in distinct sites [37]. Taken together, these results indicate that inhibition of the ETC occurs mainly at the level of the succinate-cytochrome *c* oxidase segment of ETC (i.e. bc₁ complex).

The role of sulphhydryl oxidation in the deleterious effects exerted by H₂O₂ on ETC was investigated. After incubation with H₂O₂, mitochondria were treated for 30 min with increasing concentrations of β-mercaptoethanol. Afterwards, remnant of β-mercaptoethanol and H₂O₂ were washed twice with fresh

medium to avoid the reduction of electron acceptors by β-mercaptoethanol. Also, this ensures that the effect exerted by β-mercaptoethanol can be attributed to thiol reduction and not to the H₂O₂ removal from the medium. Both succinate-DCIP oxidoreductase activity (Figure 4A) and succinate-cytochrome *c* oxidoreductase (Figure 4B) were nearly restored (89% and 95%, respectively) when concentration of β-mercaptoethanol was twice the concentration of H₂O₂ used (1.0 mM for succinate-DCIP oxidoreductase and 0.5 mM for succinate-cytochrome *c* oxidoreductase). It must be pointed out that treatment of control samples with maximal concentrations used of β-mercaptoethanol (2.0 mM for succinate-DCIP oxidoreductase activity and 1.0 mM for succinate-cytochrome *c* oxidoreductase) did not have an appreciable effect on control values of enzymatic activities (inset, Figure 4A and B). These results suggest that oxidation of thiol groups on proteins from respiratory complexes could be involved in the inhibitory effect of H₂O₂.

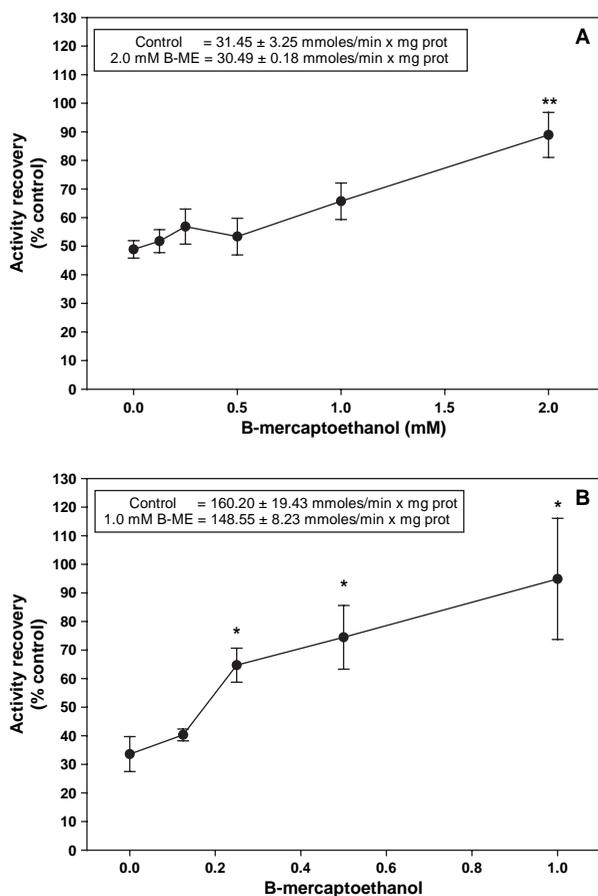


Figure 4. Effect of β -mercaptoethanol on the impairment induced by H_2O_2 on partial reactions of ETC. Mitochondria were incubated after H_2O_2 treatment with indicated concentrations of β -mercaptoethanol during 30 min at $4^\circ C$ on 50 mM KH_2PO_4 buffer. Later, H_2O_2 and β -mercaptoethanol were removed twice by centrifugation and mitochondrial pellet re-suspended on fresh 50 mM KH_2PO_4 buffer. Concentrations of H_2O_2 used were: 1.0 mM H_2O_2 for succinate-DCIP oxidoreductase activity (A) and 0.5 mM H_2O_2 for succinate-cytochrome *c* oxidoreductase activity (B). Activities were measured as described in Materials and methods. Values from mitochondria incubated with 2.0 mM β -mercaptoethanol (inset, panel A) or 1.0 mM β -mercaptoethanol (inset, panel B) without H_2O_2 addition were taken as 100%. Results are expressed as the mean \pm SE of >3 independent experiments. Significantly different when compared to control (0 mM H_2O_2) (* $p < 0.05$, ** $p < 0.01$).

H_2O_2 can also mediate oxidative damage through iron release by degrading haem proteins and iron-sulphur clusters. Released iron reacts with H_2O_2 to produce the highly reactive hydroxyl radical (OH^\bullet), which in turn oxidizes lipids and proteins [19,38,39]. To further investigate about the nature of the damage induced by H_2O_2 on ETC, the protective effect of the iron quelator EDTA and the OH^\bullet scavenger mannitol was tested. The pre-incubation during 15 min with either 10 mM mannitol or 50 μM EDTA previous to treatment with H_2O_2 did not protect both succinate-DCIP oxidoreductase activity (Figure 5A) and succinate-cytochrome *c* oxidoreductase (Figure 5B) from the inhibitory effect of H_2O_2 . Even an augment

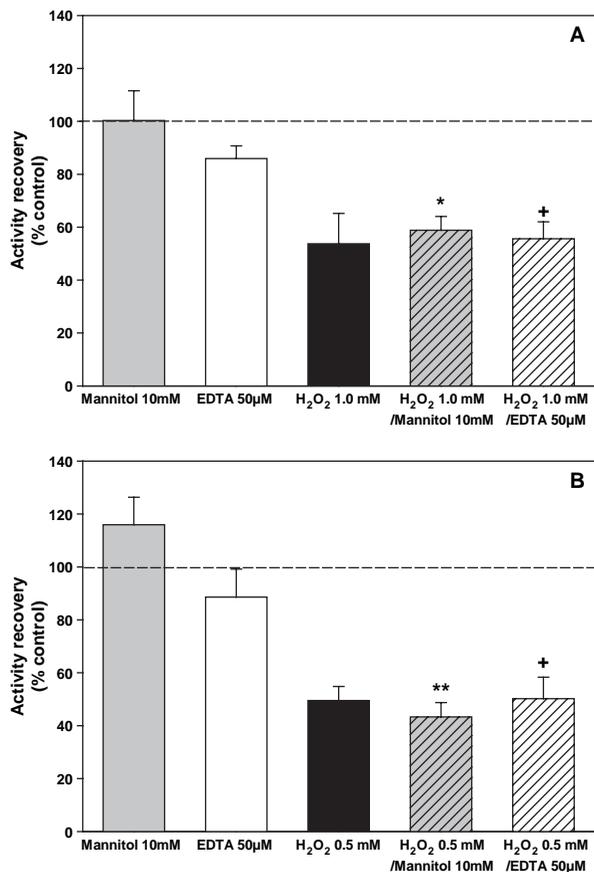


Figure 5. Effect of mannitol and EDTA on the impairment induced by H_2O_2 on partial reactions of ETC. Triton-solubilized mitochondria were incubated during 15 min on 50 mM KH_2PO_4 buffer at $4^\circ C$ with 10 mM mannitol or 50 μM EDTA previous to treatment during 30 min with 1.0 mM H_2O_2 for succinate-DCIP oxidoreductase activity (A) and 0.5 mM H_2O_2 for succinate-cytochrome *c* oxidoreductase activity (B). Activities were measured as described in Materials and methods. Values from incubated controls (mitochondria incubated during 45 min without EDTA, mannitol or H_2O_2 addition, data not shown) were taken as 100% (dotted line). Results are expressed as the mean \pm SE of >3 independent experiments. Significantly different when compared to 10 mM mannitol (* $p < 0.05$, ** $p < 0.01$) and 50 μM EDTA (⁺ $p < 0.05$).

of 10-fold in the concentration of mannitol (100 mM) did not exert protective effects (data not shown), while an augment of 2-fold (100 μM) in EDTA concentration inhibit *per se* control activities (data not shown). Thus, this result supports the suggestion that thiol oxidation is the main factor responsible for ETC inhibition and discards the participation of OH^\bullet radical in the effects observed.

Next, to locate a putative site in the bc_1 complex where H_2O_2 exerted its effects, difference absorption spectra of cytochromes were recorded. Experiments were conducted in the presence of different quinone redox sites inhibitors of bc_1 complex. The amount of reducible cytochromes *c* (550 nm peak) using dithionite was similar in controls (6.8×10^{-4} mM) (Figure 6, solid line) and in mitochondria subjected to oxidative stress (7.0×10^{-4} mM) (Figure 6, dotted

line). In addition, the content of cytochrome *b* (562 nm peak) was 3.1×10^{-4} mM in the control and 3.4×10^{-4} mM in treated mitochondria. These results suggest that the oxidative stress induced by H_2O_2 exposure did not cause the release of cytochromes from the bc_1 complex.

Antimycin A is a bc_1 complex inhibitor that prevents re-oxidation of cytochromes *b* at Q_i site, which is reflected as a decrease from 2:1 (Figure 7A, continuous lines) to ~ 1.5 :1 (Figure 7A, dotted lines) in the absorbance ratio of the reduced cytochromes *c* vs *b*. This was observed both in control mitochondria and in H_2O_2 -treated mitochondria in the presence of succinate as electron donor. Thus, H_2O_2 did not change the effect of antimycin A on bc_1 complex.

Myxothiazol is a bc_1 complex inhibitor that prevents ubiquinol oxidation at the proximal position of Q_O site. The effect of myxothiazol is reflected as a decrease from 2:1 to ~ 0.8 :1 in the absorbance ratio of reduced cytochromes *c* vs *b* (Figure 7B, continuous line). In contrast to antimycin A, H_2O_2 modified the effects of myxothiazol, inducing a full resistance to inhibition and allowing observing a ratio of 2:1 of reduced cytochromes *c* to reduced cytochromes *b* (Figure 7B, dotted line).

In order to further explore the effect of H_2O_2 on the Q_O site of cytochrome bc_1 , another Q_O site inhibitor was tested. Stigmatellin is an inhibitor that completely abolishes electron transfer at the distal niche of Q_O site. This effect can be observed in difference absorption spectra as the disappearance of the absorption peak of cytochromes *c* at 550 nm

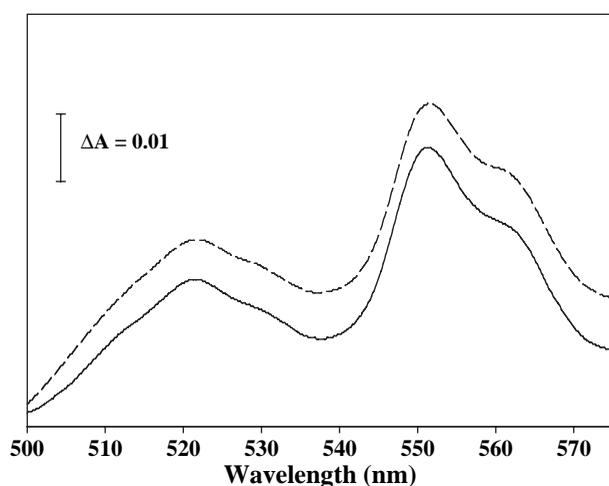


Figure 6. Effect of H_2O_2 on cytochromes levels of yeast mitochondria using dithionite as electron donor. Mitochondria were pre-incubated during 30 min on 50 mM KH_2PO_4 buffer at $4^\circ C$ in the absence (solid line) or the presence of 0.5 mM H_2O_2 (dotted line). Later, H_2O_2 was removed twice by centrifugation and mitochondrial pellet re-suspended on fresh 50 mM KH_2PO_4 buffer. A small amount of dithionite was used as electron donor. Spectra recording was performed as described in Materials and methods. Experiments were repeated at least three times. Data are from a representative experiment.

(Figure 7C, continuous line). In contrast to myxothiazol, H_2O_2 did not modify the effect of stigmatellin (Figure 7C, dotted line). Taken together, these results suggest that oxidative stress induced by H_2O_2 exposure alters selectively the proximal niche of Q_O site at complex bc_1 . The effect on the Q_O site probably is the mechanism underlying the observed decrease in the rate of electron transfer at the level of the succinate cytochrome *c* oxidoreductase region of ETC.

In mammalian mitochondria subjected to oxidative stress, lipid peroxidation has been reported as the main factor affecting ETC function [11–14]. We evaluated lipid peroxidation to explore whether it could be involved in the harmful effects of H_2O_2 on yeast ETC. Lipid peroxidation in yeast mitochondria did not rise by treatment with H_2O_2 (Figure 8) but, instead, diminished TBARS formation in 0.27 nmoles (0.74 vs 0.47 nmoles TBARS/mg of protein in control vs treated mitochondria, respectively). To verify whether H_2O_2 was an appropriate inducer of mitochondrial lipid peroxidation, mitochondria from rat liver and brain were challenged with H_2O_2 in the same manner as yeast mitochondria. First, it was observed that basal levels of lipid peroxidation were higher in rat mitochondria than yeast mitochondria. Second, H_2O_2 stimulated TBARS level increased $\sim 72\%$ in liver mitochondria (1.26 vs 2.17 nmoles TBARS/mg of protein in control vs treated mitochondria, respectively) and 61% in brain mitochondria (2.26 vs 3.64 nmoles TBARS/mg of protein in control vs treated mitochondria, respectively). These results suggest that H_2O_2 is able to induce lipid peroxidation in isolated mitochondria and that such a process may not be involved in the deleterious effects of H_2O_2 on yeast mitochondrial ETC.

Discussion

The results obtained in this study describe the effects of oxidative stress generated by H_2O_2 on yeast ETC functionality. H_2O_2 has been considered the most important ROS at cellular level [18], whereas mitochondria are the major site of H_2O_2 generation due to the presence of Mn and CuZn-SOD isoforms [18], which convert superoxide to H_2O_2 byproduct of respiration. In addition, activation of NADPH oxidases on immune cells (e.g. macrophages) increase cellular levels of H_2O_2 [40], supporting the rationale of using this oxidant as an oxidative stress inducer.

H_2O_2 decreases oxygen consumption rate in all the respiratory states (Figure 1), even in the lowest concentration used (0.125 μM). This result can be interpreted as a consequence of selective damage on some segment of ETC, as was evidenced by the full lost in activity of succinate cytochrome *c* reductase

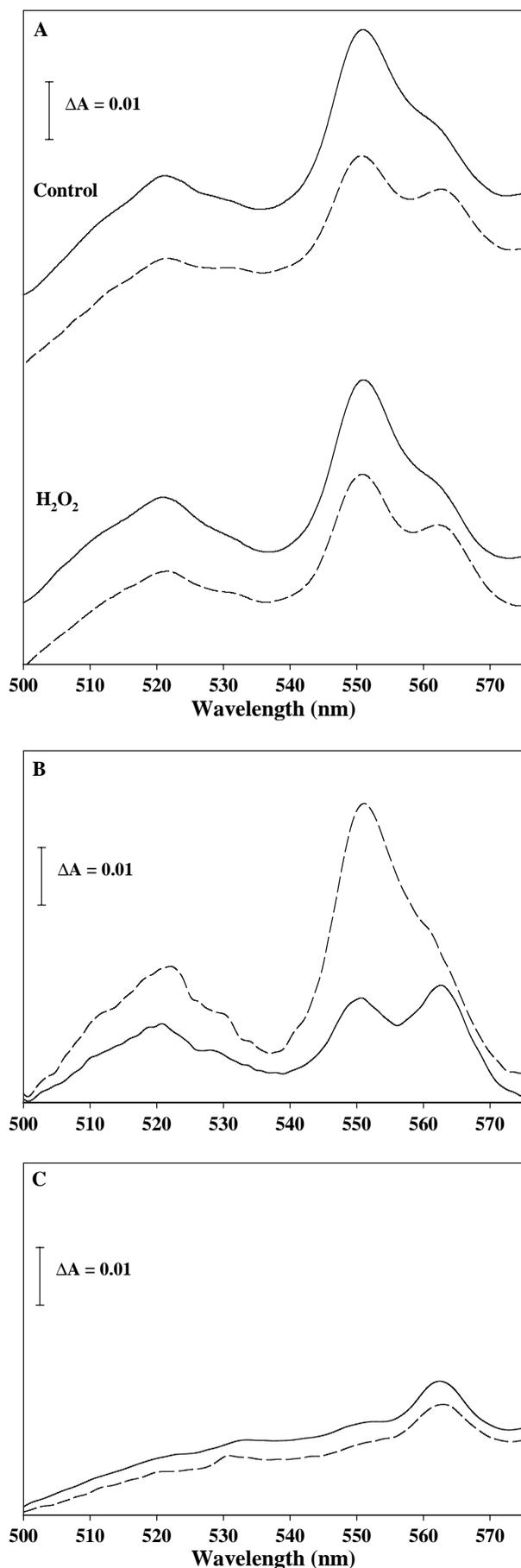


Figure 7

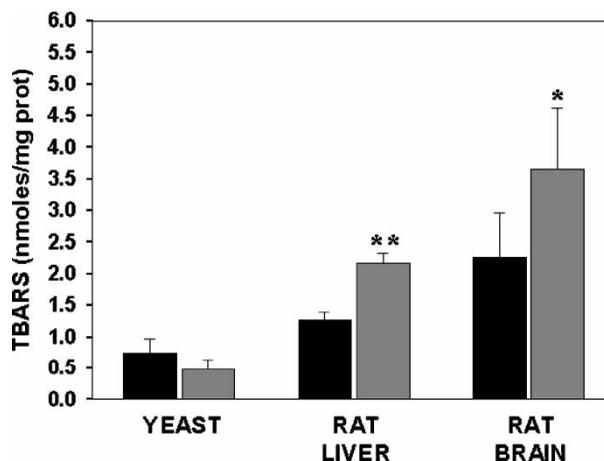


Figure 8. Effect of H_2O_2 on lipid peroxidation levels in yeast and rat mitochondria. Mitochondria were pre-incubated during 30 min on 50 mM KH_2PO_4 buffer at 4°C in the absence (black bars) or the presence (grey bars) of 0.5 mM H_2O_2 . Measurements were made as described in Materials and methods. Data are presented as mean \pm SE of three independent experiments. Significantly different when compared to yeast mitochondria treated with H_2O_2 (* $p < 0.05$, ** $p < 0.01$).

(Figure 2B). In agreement with this idea, it has been demonstrated that, due to their susceptibility of certain proteins of yeast, mitochondria undergo oxidative modifications by H_2O_2 exposure [41]. These modifications include methionine residues oxidation, irreversible $-SH$ groups oxidation, carbonylation, protein cross-linking, etc. [42]. The inhibition observed in complex II–complex III segment could be due to one of those modifications: the recovery of the activity obtained with β -mercaptoethanol (Figure 4) and the null protective effect of mannitol and EDTA on enzymatic activities suggests that oxidation of $-SH$ groups are the main event responsible for the inhibition. In addition, given the hydrophilic nature of β -mercaptoethanol, it is likely that the damage to succinate cytochrome *c* reductase was exerted in a hydrophilic region.

The resistance towards quinone redox sites inhibitors of bc_1 complex in native [43] and mutant organisms [44,45] has been used to identify these

Figure 7. Effect of H_2O_2 on difference absorption spectra of cytochromes from yeast mitochondria in the presence of bc_1 complex inhibitors. Mitochondria were pre-incubated during 30 min on 50 mM KH_2PO_4 buffer at 4°C in the absence or the presence of 0.5 mM H_2O_2 . Later, H_2O_2 was removed twice by centrifugation and mitochondrial pellet re-suspended on fresh 50 mM KH_2PO_4 buffer. Inhibitors were added 5 min before spectra recording and 10 mM succinate was used as electron donor. Spectra were performed as described in Materials and methods. (A) Spectra in absence (solid line) or the presence (dotted lines) of antimycin A, (B) spectra in the presence of myxothiazol in control (solid line) and treated (dotted line) mitochondria, (C) spectra in the presence of stigmatellin in control (solid line) and treated (dotted line) mitochondria. Experiments were repeated at least three times. Data are from a representative experiment.

sites, as well as to establish structure–activity relationships. A similar approach was used to identify which redox site in bc_1 complex underwent alterations during oxidative stress. Stigmatellin is a Q_O site competitive inhibitor that fully avoids ubiquinol oxidation at the distal position [46]. H_2O_2 did not modify the effects of this inhibitor (Figure 7C), suggesting that stigmatellin binding occurred. However, the myxothiazol-resistant cytochrome c reduction, observed in the difference spectra (Figure 7B), reflected some kind of alteration caused by H_2O_2 on the proximal niche of Q_O site. The resistance towards myxothiazol could be explained by either (a) the myxothiazol binding site was modified and thus, inhibitor binding was not possible or (b) an increase in superoxide formation was promoted, leading to the non-catalytic reduction of cytochrome c . Muller et al. [46] demonstrated that interaction of myxothiazol with the proximal niche of Q_O site allows for partial ubiquinol oxidation, but prevents the transfer of a second electron from ubisemiquinone, allowing for its accumulation and the reduction of cytochrome c through superoxide formation. Likewise, it is possible that H_2O_2 modified the environment in the proximal niche, allowing ubisemiquinone escape and non-catalytic cytochrome c reduction. Therefore, Q-cycle would suffer a diminution in its efficiency, limiting partially electron flow towards cytochrome c oxidase in the succinate cytochrome c oxidoreductase segment of the respiratory chain, as reflected by the partial decline observed in respiratory rate even at the highest concentration of H_2O_2 used.

The idea of a direct interaction between H_2O_2 and proteins of the bc_1 complex is supported by the null effects obtained with H_2O_2 on yeast mitochondria lipid peroxidation (Figure 8). In bovine heart sub-mitochondrial particles [11], rat synaptosomes [12] and rat heart mitochondria [13,14], mitochondrial lipid peroxidation has been identified as a key factor in ETC inhibition. In our hands, mitochondrial yeast lipid peroxidation did not increase due to oxidative stress while the same treatment promoted lipid peroxidation in rat liver and brain mitochondria. It is important to emphasize that conditions used in this work to induce oxidative stress were different from those used in the above reports. Even though H_2O_2 does not induce lipid peroxidation by itself, peroxides can induce this process through iron release by degrading hem proteins (e.g. cytochromes c and b) [19]. Iron reacts with H_2O_2 through Fenton's chemistry to produce the highly reactive hydroxyl radical (OH^\bullet) [38]. The latter can initiate lipid peroxidation readily through hydrogen abstraction from double bonds of phospholipids unsaturated hydrophobic chains [39]. Consequently, it is possible that H_2O_2 could have promoted lipid peroxidation by this mechanism in rat mitochondria but not in yeast mitochondria.

In agreement with this argument, different absorption spectra revealed that treatment with H_2O_2 did not diminish the content of either cytochromes c or b , since its reduction in the presence of dithionite was similar to control experiment results (Figure 6), which means that iron was not released, at least from proteins containing this sort of cytochromes (e.g. bc_1 complex). Otherwise, cytochrome reduction would not have been possible.

The unsaturation degree of fatty acids from mitochondrial membranes has been positively correlated with their vulnerability to peroxidation [24]. Fatty acid content from membranes of yeast mitochondria grown on lactate consists mainly of the monounsaturated fatty acids, palmitoleic (C16:1) and oleic (C18:1) acids [27], whereas rat mitochondria membranes contain the highly unsaturated fatty acids, docosahexaenoic acid (22:6) and arachidonic acid (20:4) [23,24]. Thus, the lower double bond content in lipids from yeast mitochondria could increase their resistance against lipid peroxidation, which would explain, in part, the differences observed in this parameter between yeast and rat mitochondria.

A contributor to the resistance of yeast mitochondria against oxidative stress could be the Hsp60 molecular chaperone. Cabiscol et al. [20] demonstrated that Hsp60 protects proteins containing Fe/S clusters from damage by H_2O_2 , avoiding in this manner the release of iron from these proteins and allowing greater cell viability than that observed in cells with low Hsp60 levels.

In summary, the results show a high resistance of yeast mitochondria against oxidative stress, as was suggested first by Machida et al. [47]. The study of complex bc_1 inhibition by oxidative stress in terms of the Q cycle dysfunction must also be considered and not only in function of lipid peroxidation and ubiquinone and cardiolipin loss as discussed above, the first could lead to a greater production of ROS. On the other hand, it would be interesting to evaluate whether the differences observed in lipid peroxidation propensity between mammalian and yeast mitochondria are involved in the dissimilarities observed in some important physiological processes where lipid peroxidation has been implicated (e.g. programmed cell death).

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