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# Mechanisms of *Saccharomyces Cerevisiae* PMA1 $H^+$-ATPase Inactivation by $Fe^{2+}$, $H_2O_2$ and Fenton Reagents

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Although considerably more oxidation-resistant than other P-type ATPases, the yeast PMA1 $H^+$-ATPase of *Saccharomyces cerevisiae* SY4 secretory vesicles was inactivated by $H_2O_2$, $Fe^{2+}$, Fe- and Cu-Fenton reagents. Inactivation by $Fe^{2+}$ required the presence of oxygen and hence involved auto-oxidation of $Fe^{2+}$ to $Fe^{3+}$. The highest $Fe^{2-}$ (100 μM) and $H_2O_2$ (100 mM) concentrations used produced about the same effect. Inactivation by the Fenton reagent depended more on $Fe^{2+}$ content than on $H_2O_2$ concentration, occurred only when $Fe^{2+}$ was added to the vesicles first and was only slightly reduced by scavengers (mannitol, Tris, $NaN_3$, DMSO) and by chelators (EDTA, EGTA, DTPA, BPDS, bipyridine, 1, 10-phenanthroline). Inactivation by Fe- and Cu-Fenton reagent was the same; the identical inactivation pattern found for both reagents under anaerobic conditions showed that both reagents act via $OH^{\cdot}$. The lipid peroxidation blocker BHT prevented Fenton-induced rise in lipid peroxidation in both whole cells and in isolated membrane lipids but did not protect the $H^+$-ATPase in secretory vesicles against inactivation. ATP partially protected the enzyme against peroxide and the Fenton reagent in a way resembling the protection it afforded against SH-specific agents. The results indicate that $Fe^{2+}$ and the Fenton reagent act via metal-catalyzed oxidation at specific metal-binding sites, very probably SH-containing amino acid residues. Deferrioxamine, which prevents the redox cycling of $Fe^{2+}$, blocked $H^+$-ATPase inactivation by $Fe^{2+}$ and the Fenton reagent but not that caused by $H_2O_2$, which therefore seems to involve a direct non-radical attack. Fe-Fenton reagent caused fragmentation of the $H^+$-ATPase molecule, which, in Western blots, did not give rise to defined fragments bands but merely to smears.

*Keywords*: Yeast; PMA1 $H^+$-ATPase; Oxidative damage; Hydrogen peroxide; Iron; Fenton reagent

*Abbreviations*: BPDS, bathophenanthroline sulphonate; BHT, butylated hydroxytoluene; DMSO, dimethylsulfoxide; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis (β-aminoethyl ether) *N,N,N′,N′*, tetraacetic acid; FITC, fluorescein isothiocyanate; MCO, metal-catalyzed oxidation; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances

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## INTRODUCTION

In cells attacked by exogenous reactive oxygen species (ROS), one of the main targets is the plasma membrane. In many cell types, membrane damage is caused by lipid peroxidation, which leads secondarily to radical assault on membrane proteins[1] while in other cells, i.e. in yeast species such as *S. cerevisiae* or *Schizosaccharomyces pombe*, the level of lipid peroxidation is low[2,3] and membrane proteins may be damaged by other mechanisms. The present study concentrates on ROS-induced inactivation of the main yeast plasma membrane protein, the PMA1 $H^+$-ATPase, which belongs to the P-type ATPases widespread in prokaryotic and eukaryotic cells. It pumps protons out of the cells to form a $\Delta\mu_{H+}$ that supplies the energy necessary for nutrient uptake. The proper functioning of the enzyme is vital for the physiology of the cell and impairment of its activity may hamper or completely abolish cell functions.

The damage of other P-type ATPases such as the $Na^+,K^+$-ATPase from rabbit kidney,[4] human red blood cells[1] and pig kidney,[5] the SR $Ca^{2+}$-ATPase from rabbit muscle[6,7] and the lobster abdominal muscle microsomes[8] by radicals generated by the Fenton reaction was in most cases proposed to involve metal-catalyzed oxidation (MCO), although membrane lipid peroxidation was considered as another possibility[1] and its role was stressed[9] in the inactivation of dog kidney $Na^+,K^-$-ATPase. Studies of the inactivation of the *S. cerevisiae* PMA1 $H^+$-ATPase have so far been mostly done with sulfhydryl agents such as *N*-ethylmaleimide, methylmethane thiosulphonate or FITC in order to define the structure-function relations in the enzymes.[10–12] We examined the response of the PMA1 $H^+$-ATPase from isolated *S. cerevisiae* SY4 secretory vesicles to the action of ROS. In this strain, which has a block in the secretory pathway, the secretory vesicles are accumulated in large amounts in the cells. They are oriented inside out, with the cytoplasmic face featuring the $H^+$-ATPase exposed to the medium and therefore accessible to externally applied oxidants.

## MATERIALS AND METHODS

### Yeast Growth and Preparation of Secretory Vesicles

Strain SY4 of *S. cerevisiae* (MATa; ura3-52; leu2-3, 112; his4-619; sec6-GAL; pmal::Y1PGAL-PMA1), kindly provided by Prof. C.W. Slayman, Yale University, carries temperature-sensitive sec6-4 mutation and a wild-type copy of the PMA1 gene under the control of the GAL1 promoter (described in detail in [13]). The sec6-4 mutation prevents the fusion of secretory vesicles with the plasma membrane and causes their accumulation in the cells. The isolation of secretory vesicles containing wild-type or mutant $H^+$-ATPase is described elsewhere.[13,14] Briefly, SY4 cells were grown for three days at 23°C on agar plates containing yeast nitrogen base, 2% galactose and 0.02% histidine. This was followed by growth to the mid-exponential phase at 23°C on liquid histidine-supplemented minimal medium containing galactose. After a temperature shift from 23 to 37°C the cells stopped dividing and became filled with vesicles. After a 2 h incubation at 37°C the cells were converted into spheroplasts by zymolyase treatment. The spheroplasts were coated with concanavalin A and spun down at low centrifugal forces to rupture the plasma membranes, remove cell debris and contaminating organelles. The vesicles remained in the supernatant and were collected by high-speed centrifugation (113,000*g*) over a two-step discontinuous sucrose gradient. The secretory vesicles located at the 37.5/20% interface of the two sucrose layers were collected and washed in 1 mM EGTA/Tris, pH 7.5, to give a crude vesicle preparation suitable for characterizing the $H^+$-ATPase activity. They were stored at −80°C in 1 mM EGTA/Tris, pH 7.5, with protease

inhibitors. The protein content of the preparation varied between 3–4 mg protein/ml.

### Measurement of Activity of Oxidant-exposed $H^+$-ATPase

Secretory vesicles stored at −80°C and allowed to thaw on ice were resuspended at 0.4 mg/ml in 50 mM HEPES-NaOH, pH 7. A 5 min preincubation at 30°C in a water bath followed after the resuspension step. $Fe^{2+}$ from a fresh aqueous 0.5 mM stock solution was added to the sample to reach the desired final concentration, followed after 30 s by $H_2O_2$. The actual concentrations of both $Fe^{2+}$ and $H_2O_2$ in the system may change somewhat due to iron autooxidation and $H_2O_2$ formation by $O_2^-$ dismutation (see Results). Unless otherwise stated, chelators and scavengers from freshly made stock solutions were supplied to the Eppendorf vial containing secretory vesicles before the addition of the Fenton reagent. At intervals, 5 µl sample aliquots were removed and diluted 100-fold in an ATPase reaction buffer that contained 50 mM MES-Tris, 5 mM $Na_2ATP$, 5 mM $NaN_3$, 5 mM phosphoenolpyruvate and 50 µg/ml pyruvate kinase. The measurement of the inorganic phosphate was done according to Fiske and Subbarow.[15]

The activity of the control (the 0 min sample) was taken as 100% and the activities of the other samples were referred to it. To each series, blanks containing the chelator and scavenger tested were run to determine their non-interference with the assay.

When testing the ATP-induced protection of the $H^+$-ATPase against inactivation, 5 or 10 mM Mg.ATP was added to the Eppendorf vial with secretory vesicles. The contents of the vial were prewarmed in a water bath at 30°C bath for 5–7 min and the reaction was started by addition of ferrous iron. All the following steps were as described above. Protein content was measured by the method of Lowry using bovine serum albumin as standard.

### Measurement of Lipid Peroxidation

Lipid peroxidation was assessed in whole cells and in lipids extracted from the purified plasma membrane fraction by methanol/chloroform by measuring the concentration of thiobarbituric acid reactive substances (TBARS) in the samples.[16]

### Measurement of $H^+$-ATPase Inactivation by $Fe^{2+}$ Under Anaerobic Conditions

The procedure was the same as above, the experiments being done in the Coy Anaerobic Chamber (Toepffer Labortechnik, Göppingen, Germany) and involving the use of deaerated solutions.

### Gel Electrophoresis

SDS-PAGE was done according to Laemmli.[17] The SDS gels were composed of 4% stacking gels and 10% separating gels. The control sample consisted of 20 µg protein added to an aliquot that contained 50 mM HEPES pH 7 and double-distilled water to give a total volume 100 µl, other samples were supplied with appropriate concentrations of $Fe^{2+}$, hydrogen peroxide or the Fenton reagent. All samples were then incubated for 30 min at room temperature. Subsequently, 900 µl of 15% TCA was added to each aliquot and the samples were incubated on ice for 30 min and then centrifuged for 30 min at 4°C at 14,000 rpm. The supernatant was discarded, 400 µl of 80% acetone was added to each vial and the samples were centrifuged at 14,000 rpm for another 20 min. Acetone was removed, a volume of 20–25 µl of SDS buffer with dithiothreitol prewarmed at 30°C was added to each sample and the pellet was resuspended by vortexing. The samples were then loaded onto the gel and the gel was run at 100 V for 2 h. The intensity of the bands in the gels was assessed by the UN-SCAN-IT Automated Digitizing System, version 4.1 (Silk Scientific Corporation).

### Western Blot

The gel was removed from the glass plate and carefully transferred to a nitro-cellulose membrane. Special care was taken to avoid air bubbles that remain trapped between the gel, the membrane and the filter paper. The electrophoresis was run on the Bio-Rad Western blot device at 4°C at 100 V for 2 h or at 50 V overnight. The $H^+$-ATPase was visualized by overnight incubation with specific polyclonal antibodies kindly provided by Prof. C.W. Slayman, Yale University.

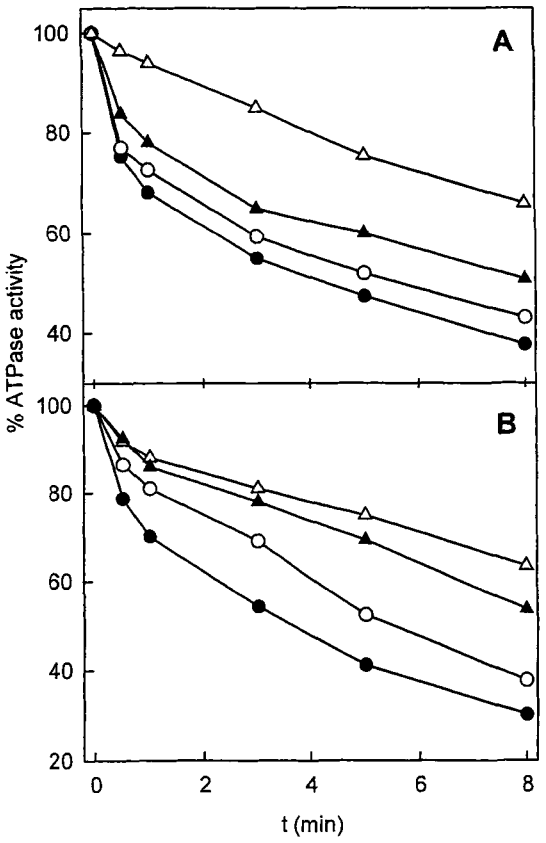


FIGURE 1 Time course of inactivation of $H^+$-ATPase in isolated secretory vesicles from *S. cerevisiae* by (A) 20 (△), 50 (▲) 80 (○) and 100 (●) mM $H_2O_2$ and by (B) 20 (△), 50 (▲), 80 (○) and 100 (●) μM $Fe^{2+}$. The data are means of three independent measurements.

## RESULTS

### Inactivation of the $H^+$-ATPase by $Fe^{2+}$, $H_2O_2$ and the Fenton Reagent

To most animal cells, $H_2O_2$ is toxic in concentrations of 10–100 μM. Exponential-phase yeast cells are lethally damaged by millimolar $H_2O_2$[18,19] whereas stationary cells can tolerate up to 100 mM $H_2O_2$.[20,21] As shown in Fig. 1A,B, the $H^+$-ATPase in the *S. cerevisiae* SY4 secretory vesicles isolated from mid-exponential phase cells is fairly resistant to $H_2O_2$ since its inactivation on an 8 min exposure to 20–100 mM $H_2O_2$ is in the range of 34–62%.

As seen in Fig. 1B, $Fe^{2+}$ alone inactivated the $H^+$-ATPase in the secretory vesicles. The loss of activity caused by an 8 min exposure to 20–100 μM $Fe^{2+}$ was comparable with that caused by 20–100 mM $H_2O_2$. This inactivating effect of $Fe^{2+}$ may involve two mechanisms: (a) $Fe^{2+}$ binds to the enzyme and inactivates it by itself, probably by forming chelates with some as yet unknown physiologically important sites. This action would not require the participation of another agent such as oxygen; (b) the $Fe^{2+}$ added to the

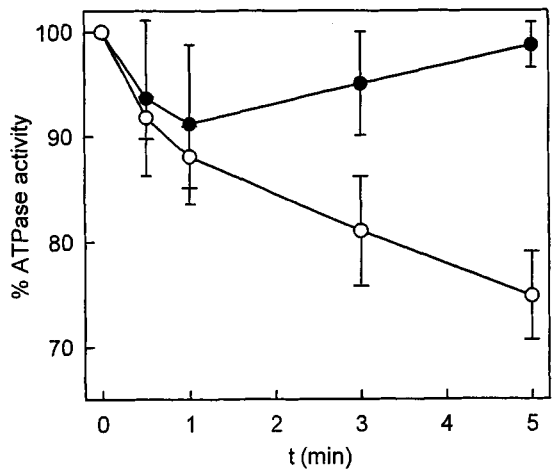


FIGURE 2 Lack of inactivation of $H^+$-ATPase in isolated secretory vesicles by $Fe^{2+}$ in the absence of oxygen. (●) anaerobic conditions, (○) aerobic conditions. The data are means of three independent measurements ± SD.

sample reacts with oxygen to yield $O_2^{\cdot-}$.[22]

$$Fe^{2+} + O_2 \rightarrow Fe^{2+} - O_2 \rightarrow Fe^{3+} + O_2^{\cdot-}$$

The superoxide radical can then damage the enzyme, or it may dismutate to hydrogen peroxide, which reacts with the enzyme-bound $Fe^{2+}$ to yield the reactive $OH^{\cdot}$. The damaging action of $O_2^{\cdot-}$ is improbable since in experiments performed in the presence of added superoxide dismutase (data not shown) the enzyme had no effect on the extent of the $Fe^{2+}$-induced inactivation.

If the above reaction occurs, then the key element in it must be oxygen. $H^+$-ATPase inactivation tests with $Fe^{2+}$ alone were therefore performed in the presence and absence of oxygen. Figure 2 shows that in the absence of oxygen $Fe^{2+}$ exerts hardly any inactivating action on the enzyme. Inactivation thus occurs only when oxygen is available in the experimental solutions and in the ambient atmosphere. These results indicate that the above reaction sequence is the probable mechanism of $H^+$-ATPase inactivation by $Fe^{2+}$.

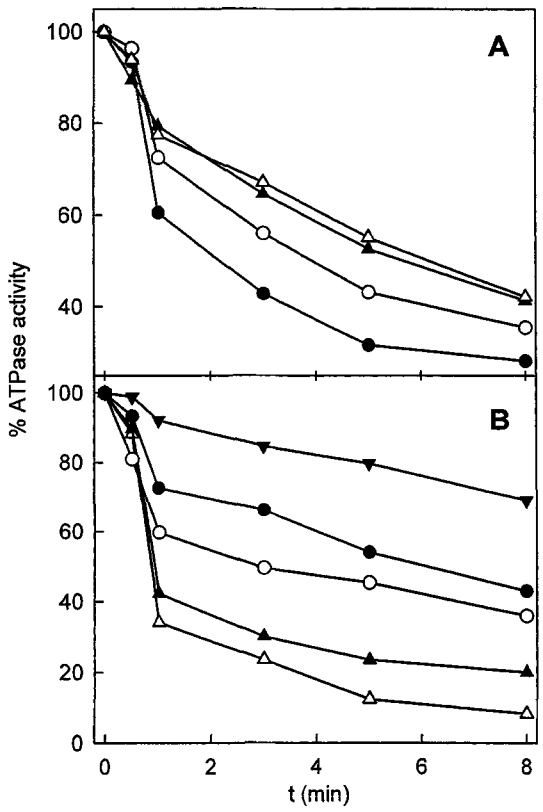


FIGURE 3 Time course of inactivation of $H^+$-ATPase in isolated secretory vesicles from *S. cerevisiae* by (A) the Fenton reagent containing a fixed concentration (50 μM) of $Fe^{2+}$ and 10 (△), 20 (▲), 50 (○) and 100 (●) mM $H_2O_2$ and by (B) the Fenton reagent containing a fixed concentration (20 mM) of $H_2O_2$ and 20 (▼), 50 (●), 80 (○), 100 (▲) and 200 (△) μM $Fe^{2+}$.

Exposure of the secretory vesicles to the Fenton reagent, which generates $OH^{\cdot}$ radicals, inactivated again the enzyme (Fig. 3A,B). As found previously with the $H^+$-ATPase in the isolated plasma membrane fraction from *S. pombe*,[20] the inactivation occurred only when the addition of $Fe^{2+}$ to the $H^+$-ATPase containing secretory vesicles preceded the addition of $H_2O_2$. When the concentration of $H_2O_2$ was kept fixed at 20 mM and the concentration of $Fe^{2+}$ was increased, the $H^+$-ATPase inactivation became progressively more severe. Most of the damage was done in the first minute of incubation with the Fenton reagent (Fig. 3A). The figure shows that, at the excess of $H_2O_2$ used in the Fenton system, the amount of available $Fe^{2+}$ is crucial for the formation of the $OH^{\cdot}$ radicals. The residual $H^+$-ATPase activity after a 5 min incubation with the Fenton reagents was 80% of the control with 20 mM $H_2O_2$/20 μM $Fe^{2+}$ and a mere 10% with 20 mM $H_2O_2$/100 μM $Fe^{2+}$. At a fixed $Fe^{2+}$ concentration of 50 μM, increasing amounts of $H_2O_2$ brought about only a small increase in the rate and extent of the $H^+$-ATPase inactivation by the Fenton reagent (Fig. 3A). Hence increasing the excess of $H_2O_2$ in the agent has only a mild effect on the inactivation process.

Further experiments were carried out with 50 μM $Fe^{2+}$, 20 mM $H_2O_2$ and 50 μM $Fe^{2+}$/20 mM $H_2O_2$ since the inactivation caused by these concentrations was substantial (30–50% after 8 min) but not overly severe and the reproducibility of the data was satisfactory.

### Inactivation by Fe- and Cu-Fenton Reagents under Aerobic and Anaerobic Conditions

The production of $OH^{\cdot}$ may also take place in mixtures of $H_2O_2$ with transition metal ions in their higher oxidation states ($Fe^{3+}$, $Cu^{2-}$); these, however, yield another ROS, $HO_2^{\cdot}$, which may attack biomolecules.[22] In our experiments, inactivation of the $H^+$-ATPase by Fe-containing Fenton reagent was essentially identical with that brought about by Cu-containing Fenton reagent although the latter contained the oxidized form of copper, $Cu^{2+}$. The production of $OH^{\cdot}$ by the two reagents therefore involves different reaction sequences and the identical inactivation pattern might be coincidental. To prove the essential identity of action of Fe-Fenton and Cu-Fenton reagent we measured the $H^+$-ATPase inactivation by both of them under both aerobic and anaerobic conditions. As seen from the results (Fig. 4), the $H^+$-ATPase damage by both agents was virtually identical even under anaerobic conditions.

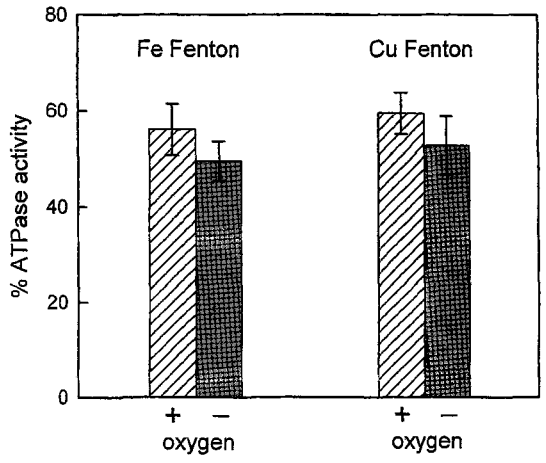


FIGURE 4 Inactivation of the $H^+$-ATPase by Fe-Fenton and Cu-Fenton under aerobic and anaerobic conditions. Fe-Fenton—50 μM $Fe^{2+}$/20 mM $H_2O_2$, Cu-Fenton—50 μM $Cu^{2+}$/20 mM $H_2O_2$. Hatched columns-aerobic conditions, dark columns-anaerobic conditions. The data for aerobic experiments are means ± SD from 14 and six independent measurements, respectively, those for anaerobic ones are means of three measurements each.

### Protection of $H^+$-ATPase by Chelators and Radical Scavengers

Possible differences between the effect of free and chelated iron on the $H^+$-ATPase inactivation were explored by using a number of chelators including EGTA, EDTA, DTPA, BPDS, bipyridine, *o*-phenanthroline and deferrioxamine (Desferal). Some chelators such as EDTA have been found to increase considerably the catalytic effect of iron on the Fenton reaction since they maintain the $Fe^{3+}$ arising by $Fe^{2+}$ oxidation in a form which retains an aquo coordination site that allows redox cycling of the iron.[23,24] In our experiments, all these chelators slightly reduced $H^+$-ATPase inactivation, none of them exhibiting any augmentation of the Fenton reaction.

Other chelators, which form hexadentate chelates, such as deferrioxamine, occupy all six coordination sites and prevent $Fe^{3+}$ redox cycling and $H^+$-ATPase inactivation. As expected, deferrioxamine proved to be the only chelator efficient in protecting the enzyme against a radical damage (Table I). Its efficiency in preventing $H^+$-ATPase inactivation by preventing the $Fe^{2+}$ from undergoing the Fenton reaction documents the important role of iron in the process.

TABLE I Protective effect of chelators and radical scavengers against $H^+$-ATPase inactivation caused by a 10 min exposure of secretory vesicles to the Fenton reagent (50 μM $Fe^{2+}$/20 mM $H_2O_2$). The data are means of three measurements

| Chelators | Concentration (mM) | Protection afforded (%) |
|---|---|---|
| EGTA | 0.5 | 10 |
| DTPA | 5 | 10 |
| EDTA | 0.5 | 15.6 |
| BPDS | 0.5 | 5.4 |
| Bipyridine | 1 | 7.3 |
| 1,10-Phenanthroline | 1 | 0 |
| Deferrioxamine | 0.5 | 45 |
| | 5 | 94 |
| Scavengers | | |
| Mannitol | 50 | 0 |
| Tris | 10 | 21 |
| $NaN_3$ | 1 | 0 |
| DMSO | 1 | 0 |

Table I also shows that water-soluble radical scavengers such as mannitol, Tris, sodium azide and DMSO were not able to prevent the damage to the enzyme by the Fenton reagent. These results suggest that generation of radical species that leads to $H^+$-ATPase inactivation takes place at metal-binding sites in the protein rather than in the bulk solution. This result supports the notion that the inactivation of the PMA1 $H^+$-ATPase by the Fenton reagent is a metal-catalyzed protein oxidation.[25,26]

## Protection Afforded by ATP

Nucleotides have been found to afford protection against $H^+$-ATPase inactivation by sulfhydryl agents such as NEM by binding to the catalytic site of the enzyme.[11] This probably brings about a conformational change that will make attack-vulnerable sites in the vicinity of the ATP-binding site inaccessible to the inactivating agents. In order to find out whether nucleotide binding protects the $H^+$-ATPase also against $Fe^{2+}$- and oxidant-induced damage, we preincubated the secretory vesicles with 5 or 10 mM Mg.ATP before adding the inactivating agents. The level of protection by 5 mM Mg.ATP was 44%, by 10 mM Mg.ATP 64% (Fig. 5). This again indicates that the damage to the enzyme occurs at specific site(s) situated in the neighborhood of the ATP-binding site.

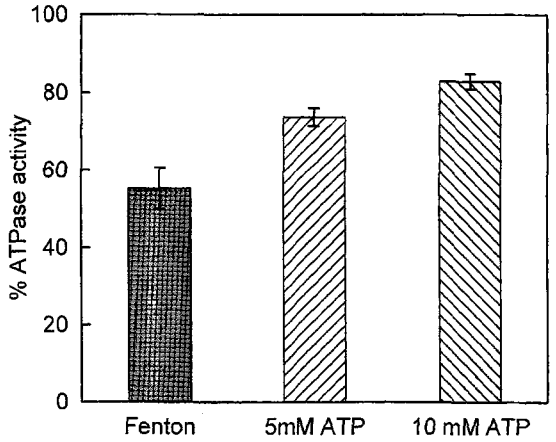


FIGURE 5 Protection afforded by 5 and 10 mM Mg.ATP against the inactivation of the $H^+$-ATPase by Fenton reagent. The secretory vesicles were incubated for 5 min with Fenton reagent (50 μM $Fe^{2+}$/20 mM $H_2O_2$) after a 5–7 min preincubation in pure buffer (column 1), in buffer containing 5 mM Mg.ATP (column 2) or in buffer containing 10 mM Mg.ATP (column 3). The data are means of three independent measurements.

## Lack of Protection by a Lipid Peroxidation Blocker

To document the possible role of lipid peroxidation in oxidative $H^+$-ATPase damage, we exposed the enzyme to the Fe-Fenton reagent in the presence and absence of the lipid peroxidation blocker BHT. The efficiency of its action

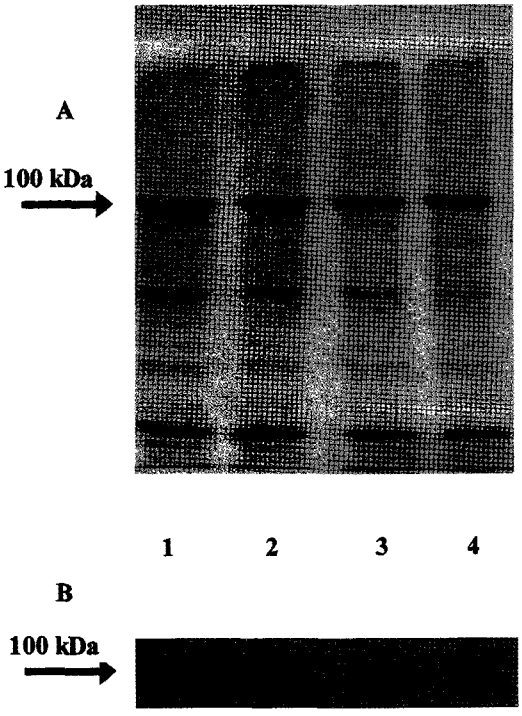


FIGURE 6 SDS-PAGE (A) and a Western blot (B) of the PMA1 $H^+$-ATPase from *S. cerevisiae* SY4 secretory vesicles exposed to increasing concentrations of the Fenton reagent. For the conditions see Materials and Methods. In both figures, the lanes are as follows: (1) control-ATPase without Fenton; (2) ATPase exposed to 50 μM $Fe^{2+}$/20 mM $H_2O_2$; (3) ATPase exposed to 100 μM $Fe^{2+}$/20 mM $H_2O_2$; (4) ATPase exposed to 200 μM $Fe^{2+}$/40 mM $H_2O_2$.

TABLE II Lowering of lipid peroxidation in intact cells and in isolated plasma membrane lipids of *S. cerevisiae* SY-4 by 50 μM BHT and the lack of protective effect against $H^+$-ATPase inactivation in secretory vesicles caused by a 10 min exposure to the Fenton reagent (50 μM $Fe^{2+}$/20 mM $H_2O_2$). The data are means of three measurements. The TBARS values in brackets are in per cent of oxidant-free, BHT—free control, nd—not determined

| Treatment | Lipid peroxidation | | | | | |
|---|---|---|---|---|---|---|
| | Whole cells (nmol TBARS/mg protein) | | Isolated membrane lipids (nmol TBARS/mg ipid) | | ATPase activity (%) | |
| | −BHT | +BHT | −BHT | +BHT | −BHT | +BHT |
| No oxidants | 1.7 (100) | nd | 560 (100) | nd | 100 | nd |
| Fenton reagent | 2.9 (171) | 1.8 (106) | 752 (134) | 432 (77) | 59 | 49 |

on lipid peroxidation in this yeast was tested by exposing whole cells and purified lipids from the plasma membrane to the Fenton reagent in the presence and absence of BHT and measuring the content of TBARS (Table II). BHT eliminated the oxidant-induced rise in TBARS levels in both intact cells and in purified membrane lipids but it had no protective effect on the $H^+$-ATPase of the secretory vesicles (Table II).

### SDS-PAGE and Western Blot Analysis of the $H^+$-ATPase Damage

The oxidative inactivation to the enzyme may be accompanied by structural damage that may give rise to fragmentation and/or aggregation of the enzyme molecules. To test for the presence of these changes, we performed SDS-PAGE and immunoblotting (Fig. 6). In both Coomassie blue-stained gels and immunoblots, the intensity of the band corresponding to the $H^+$-ATPase diminished with increasing concentration of the Fenton reagent. Although this diminishing intensity of the $H^+$-ATPase band indicates oxidative fragmentation of the molecule, no clear bands corresponding to enzyme fragments can be distinguished, the blots featuring mere diffuse "smears". The same phenomenon was previously found with other membrane enzymes (cf. Ref. [25]).

## DISCUSSION

Though it belongs to the same group of P-type ATPases as $Na^+$,$K^+$-ATPase, or $Ca^{2+}$-ATPase, the $H^+$-ATPase of the yeast plasma membrane differs from them by being much more resistant to oxidative attack. Thus the damage inflicted on the SR $Ca^{2+}$-ATPases by 5 μM $Fe^{2+}$/1 mM $H_2O_2$ plus 1 mM ascorbate[6] is about the same as that caused to the *S. cerevisiae* $H^+$-ATPase by 50 μM $Fe^{2+}$/20 mM $H_2O_2$. The resistance of the *S. pombe* $H^+$-ATPase is comparable to that of the *S. cerevisiae* enzyme.[20] In view of this difference in sensitivity to oxidant attack it was desirable to know if the mechanisms of $H^+$-ATPase inactivation are analogous to those in animal cells. The resistance of yeast cells to oxidants in terms of cell survival is much higher in stationary-phase cells, which can withstand 100 mM $H_2O_2$,[21] than in exponential ones for which millimolar $H_2O_2$ is lethal.[19,22] Our results with the oxidative inactivation of the PMA1 $H^+$-ATPase in secretory vesicles isolated from mid-exponential cells indicate that the enzyme keeps its relatively low sensitivity to oxidative inactivation even in exponential cells and thus does not participate in the high overall sensitivity of these cells to oxidants.

The lipid peroxidation blocker BHT prevented the Fenton reagent-induced rise in the levels of lipid peroxidation products in both intact cells and

in purified membrane lipids. However, it had no protective effect on the $H^+$-ATPase of the secretory vesicles, and in fact it sometimes seemed to lower the activity of the enzyme somewhat relative to the control. Hence, lipid peroxidation does not seem to play any significant role in the oxidative inactivation of the enzyme.

The enzyme is strongly inactivated by $Fe^{2+}$ alone, albeit only in the presence of ambient oxygen, and the inactivation therefore very probably involves auto-oxidation of $Fe^{2-}$ with concomitant production of $O_2^-$ and $OH^{\cdot}$.[24] The inactivation is not alleviated in the presence of a high excess of added radical scavengers and/or most iron chelators, and therefore exhibits features of MCO, a site-specific process that involves the interaction between protein-bound $Fe^{2+}$ and an oxygen species capable of forming radicals. MCO is known to be insensitive to the presence of chelators in the bulk phase[26,27] and also to the action of scavengers[28,29] due to its spatially confined "caged" generation of radicals at the metal-binding sites and their direct attack on targets in the immediate vicinity of the site of origin.

The inactivation of the $H^+$-ATPase in isolated *S. cerevisiae* secretory vesicles by the Fenton reagent was also shown to involve MCO. This conclusion is supported by the following findings: (a) the concentration of iron in the Fenton reagent plays an important role in the extent of the damage whereas the concentration of $H_2O_2$, which is in all cases in excess relative to iron, plays smaller role; (b) the extent of inactivation is not perceptibly affected, either negatively or positively, by high concentrations of chelators of various denticity, with the exception of deferrioxamine (see below); (c) classical free radical scavengers such as mannitol, Tris, sodium azide or DMSO also have no protective effect. Although the scavengers were used in large excess relative to the concentration of the Fenton reagent, still larger concentrations of scavengers (of the order of molar) would obviously be necessary to trap effectively the radicals before they reach their target on the protein;[30] (d) Mg.ATP protected the $H^+$-ATPase against oxidant-induced inactivation. This result is in keeping with the data from ATP protection studies done on the SR $Ca^{2+}$-ATPase[7] and on the vacuolar $H^+$-ATPase from brain synaptic vesicles[31] and suggests that the radical induced damage may occur to some extent at the active site of the enzyme.

Some information about the identity of the actual enzyme-inactivating species can be obtained by comparing the effect of Fe-Fenton reagent and Cu-Fenton reagent. The Fe-Fenton reagent contains the reduced form of iron, $Fe^{2+}$, whereas the Cu-Fenton reagent as commonly used contains the oxidized form copper, $Cu^{2+}$. The production of $OH^{\cdot}$ by the two reagents therefore differs: the basic reaction taking place in the Fe-containing Fenton reagent is $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$,[27] whereas the Cu-Fenton reagent containing $Cu^{2+}$ gives rise to the $OH^{\cdot}$ by a more complex reaction sequence involving $O_2$, $HO_2$ and $O_2^-$.[32] We wanted to find out whether these ROS involved in $OH^{\cdot}$ generation by the Cu-Fenton reagent can be considered mere reaction intermediates produced and then consumed during the reaction sequence, or if, for instance, the $OH^{\cdot}$ generation would require participation of ambient $O_2$ from the atmosphere or the solutions used (as found in the case of the effect of $Fe^{2+}$ alone). In the former case the two reagents would produce $OH^{\cdot}$ as the inactivating ROS irrespective of the complexity of reactions leading to it, whereas in the latter there would be a substantial difference in the respective reaction patterns and the actions of the two agents could not be taken as identical. The identical inactivation pattern found for both reagents under anaerobic conditions shows that both reagents exert their damaging effects by giving rise to $OH^{\cdot}$ radicals, the reaction intermediates playing, if any, only a marginal role in the attack on the $H^+$-ATPase molecule.

Even when added alone, deferrioxamine, a hexadentate chelator that greatly accelerates

the auto-oxidation of $Fe^{2+}$ to $Fe^{3+}$ and prevents redox cycling of iron by binding to all six coordination sites of $Fe^{3+}$, completely blocked the inactivation of the enzyme by $Fe^{2+}$ and the Fenton reagent but had no effect on the inactivation brought about by $H_2O_2$ alone. This indicates that hydrogen peroxide inactivates the $H^-$-ATPase by a different mechanism, very probably by a direct nonradical attack.[25,31] A mixture of deferrioxamine and catalase completely abolished the inactivation by the Fenton reagent.

SDS-PAGE analysis of the samples treated with various concentrations of the Fenton reagent has shown a decrease in the intensity of the 100 kDa band that corresponds to the $H^+$-ATPase. The loss in the band intensity, as assessed using the UN-SCAN-IT software, was approximately 6% for 50 μM $Fe^{2+}$/20 mM $H_2O_2$, 11% for 100 μM $Fe^{2+}$/20 mM $H_2O_2$ and 32% for 200 μM $Fe^{2+}$/40 mM $H_2O_2$. This results hints at an oxidative fragmentation of the $H^+$-ATPase molecule. We did not detect any new distinct lower-molecular-weight bands that may emerge as the result of the fragmentation, but, rather, a "smear" on the immunoblot; this "smearing", which has also been reported by other authors,[25,33,34] may be explained by the small size of these fragments.

In conclusion, we may state that, despite its much higher resistance to oxidants as compared with animal P-type ATPases, the yeast PMA1 $H^+$-ATPase is inactivated by the same mechanisms. The difference in sensitivity may be ascribed to, e.g. different composition of the lipid membrane matrix, or to differences in the conformation of the enzyme molecule, which make the target sites less accessible for oxidants. The vitally important membrane enzyme of this unicellular microorganism is thus better equipped for surviving possible changes in environmental conditions including external oxidant attack than its animal counterparts, which operate in a much more protected environment.

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