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Iron-induced oxidative damage of corn root plasma membrane H^+-ATPase

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Abstract

The effect of iron on the activity of the plasma membrane H^+-ATPase (PMA) from corn root microsomal fraction (CRMF) was investigated. In the presence of either Fe^{2+} or Fe^{3+} (100–200 μM of $FeSO_4$ or $FeCl_3$, respectively), 80–90% inhibition of ATP hydrolysis by PMA was observed. Half-maximal inhibition was attained at 25 μM and 50 μM for Fe^{2+} and Fe^{3+}, respectively. Inhibition of the ATPase activity was prevented in the presence of metal ion chelators such as EDTA, deferoxamine or *o*-phenanthroline in the incubation medium. However, preincubation of CRMF in the presence of 100 μM Fe^{2+}, but not with 100 μM Fe^{3+}, rendered the ATPase activity (measured in the presence of excess EDTA) irreversibly inhibited. Inhibition was also observed using a preparation further enriched in plasma membranes by gradient centrifugation. Addition of 0.5 mM ATP to the preincubation medium, either in the presence or in the absence of 5 mM $MgCl_2$, reduced the extent of irreversible inhibition of the H^+-ATPase. Addition of 40 μM butylated hydroxytoluene and/or 5 mM dithiothreitol, or deoxygenation of the incubation medium by bubbling a stream of argon in the solution, also caused significant protection of the ATPase activity against irreversible inhibition by iron. Western blots of CRMF probed with a polyclonal antiserum against the yeast plasma membrane H^+-ATPase showed a 100 kDa cross-reactive band, which disappeared in samples previously exposed to 500 μM Fe^{2+}. Interestingly, preservation of the 100 kDa band was observed when CRMF were exposed to Fe^{2+} in the presence of either 5 mM dithiothreitol or 40 μM butylated hydroxytoluene. These results indicate that iron causes irreversible inhibition of the corn root plasma membrane H^+-ATPase by oxidation of sulfhydryl groups of the enzyme following lipid peroxidation.

Keywords: H^+-ATPase; Plasma membrane; Iron; Oxidative stress; Plant

1. Introduction

Many essential physiological processes in plants, including the uptake of soil metabolites, stoma opening, control of cellular turgor and hormone-dependent proliferation, are dependent on the H^+ gradient generated by a H^+-ATPase inserted in the plasma membrane (PMA) [1–3]. This enzyme belongs to the P-type ATPase family, whose members share,

Abbreviations: CRMF, corn root microsomal fraction; PMA, plasma membrane H^+-ATPase; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; BHT, butylated hydroxytoluene; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; TBA, thiobarbituric acid; MCO, metal catalyzed oxidation

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among other features, a characteristic phosphorylation of a conserved aspartic residue at the catalytic site, which mechanistically couples ATP splitting to ion pumping across the membrane (for a recent review, see [4]).

At least 10 isoforms of the H^+-ATPase have been identified in plants and their distribution among different plant tissues and species has been determined [4–7]. In roots, active uptake of several soil nutrients is accomplished by transport systems dependent on the proton gradient maintained by the H^+-ATPase [1]. The root plasma membrane is also the site of absorption of various minerals, among which iron, an important co-factor for electron transfer and redox enzymes. Different iron uptake systems have been developed by plants [8,9]. The concentration of iron in the soil may vary naturally or due to environmental pollution. Iron overload may be harmful to the plant, as iron is a well-known catalyzer of the production of reactive oxygen species (ROS), which are potent oxidants of several organic molecules [10]. Cell damage observed following ROS generation under different conditions has been attributed in part to interaction of these free radicals with ion transport mechanisms [11].

In the present report, we characterize the inhibition of the plasma membrane H^+-ATPase (PMA) from corn root by iron. We show that, depending on the experimental conditions, the H^+-ATPase may be either reversibly or irreversibly inhibited by iron, and that irreversible inhibition is mediated by oxidative damage of the H^+-ATPase, involving lipid peroxidation and oxidation of enzyme sulfhydryl groups.

2. Materials and methods

2.1. Corn root microsomal fraction (CRMF) preparation

Corn (*Zea mays*) seeds were germinated in the dark at 28°C on wet filter paper. The roots were harvested on the fourth day of growth. Radicles (100 g) were cut into small pieces and homogenized three times in a Waring blender for 5 s each with 2 vols. of cold buffer containing 250 mM Tris, pH 8.0, 30% (w/v) sucrose, 25 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 0.2% (w/v) bovine serum albumin (BSA), 1.5 mM phenylmethylsulfonyl fluoride, 0.6% (w/v) PVP 40. The homogenate was filtered through eight layers of cheesecloth and centrifuged at $8000 \times g$ at 4°C for 10 min. The pellet was discarded and the supernatant was centrifuged at $100\,000 \times g$ at 4°C for 40 min. The final pellet was resuspended to approx. 10 mg/ml of protein in 10 ml of ice-cold buffer containing 20% (v/v) glycerol, 1 mM DTT, 10 mM Tris–HCl pH 7.6, 1 mM EDTA. After homogenization with six strokes in a tight-fitting glass/Teflon homogenizer [12], this suspension was frozen in liquid nitrogen and stored at −70°C until use. For some experiments (see Section 3), corn root plasma membrane was further purified using sucrose gradient centrifugation as described by Serrano [12]. Protein concentration was determined by the method of Lowry [13] using BSA as the standard.

2.2. ATP hydrolysis

This was determined by measuring the release of P_i, either colorimetrically [14] or by the $[^{32}P]P_i$ released after adsorption of non-hydrolyzed [γ-^{32}P]ATP on activated charcoal [15]. The reaction was started by the addition of either 30 μg/ml of protein or 2 mM ATP (as indicated in the legends to the figures). After 30 min of incubation at 37°C the reaction was quenched by addition of trichloroacetic acid to a final concentration of 10% (w/v). The standard reaction medium contained 50 mM BTP, pH 6.0, 50 mM KNO_3, 10 mM $MgCl_2$, 2 mM NaN_3, in the presence or in the absence of 1 mM Na_3VO_4, an inhibitor of the H^+-ATPase. The vanadate-sensitive ATP hydrolytic activity was attributed to the H^+-ATPase.

2.3. Assay of lipid peroxidation

Lipid peroxidation was measured as generation of thiobarbituric acid (TBA) reactive substances (TBARS) (based on [16]). Briefly, 0.2 ml of TBA solution (8 mg/ml in glacial acetic acid/H_2O 1:1) was added to 0.1 ml of sample. This mixture was cooled in ice for 60 min, and subsequently heated at 98°C for 30 min in a dry bath before centrifugation in an Eppendorf centrifuge. The absorbance of the supernatant was measured at 532 nm.

2.4. Western blot analysis

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was performed using a 4% acrylamide stacking gel and 8% acrylamide separating gel. Twenty micrograms of total CRMF protein were loaded per lane. Following electrophoresis, the wet slab gel was placed on a nitrocellulose membrane, and the proteins were electroblotted (190 mV) for 90 min at 4°C in transfer buffer containing 25 mM Tris–glycine, 20% (v/v) methanol, pH 8.3 [17]. Blots were blocked for 60 min at room temperature with 5% (w/v) non-fat dried milk with 0.01% (w/v) Tween 20 dissolved in 10 mM Tris–HCl, pH 7.6, 0.15 M NaCl. A polyclonal antibody against the yeast plasma membrane H^+-ATPase (used at 1/1000 dilution, and kindly provided by Dr. M. Montero-Lomeli) was incubated with the blot for 60 min at room temperature. After four washes (5 min each), the secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG from Sigma, used at 1/1000 dilution) was incubated with the membrane for 60 min with gentle stirring at room temperature. The membrane was washed with the above described buffer, and developed with a solution containing 0.03% (w/v) NBT, 0.015% (w/v) BCIP, 10 mM Tris–HCl, pH 9.6, 0.1 M NaCl, 5 mM $MgCl_2$.

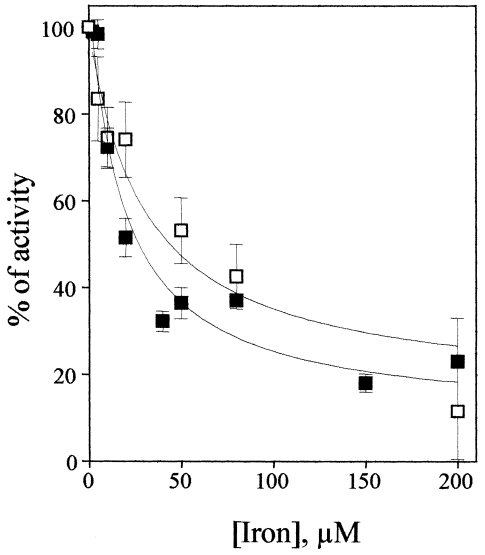

Fig. 1. Inhibition of H^+-ATPase activity by Fe^{2+} (■) and Fe^{3+} (□). The ATPase activity assay was initiated by addition of CRMF (30 μg/ml) to standard reaction medium (see Section 2) containing the indicated concentrations of iron. Data represent averages ± S.D. of experiments carried out using at least six different preparations. The control activity (100%) corresponded to 200 nmoles P_i/mg·min.

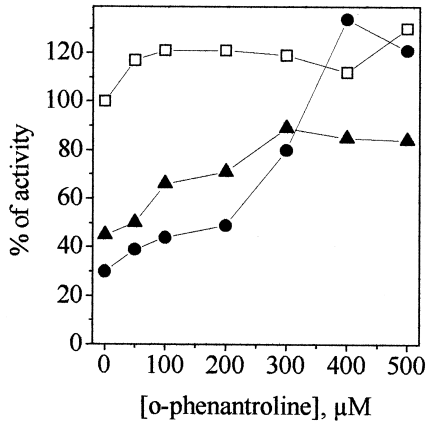

Fig. 2. Inhibition of the H^+-ATPase activity by iron is prevented by metal ion chelators. The experimental conditions were as described in the legend to Fig. 1. Effect of *o*-phenanthroline on ATPase activity in the absence (□) or in the presence of Fe^{2+} (100 μM, ●) or Fe^{3+} (100 μM, ▲).

3. Results

3.1. Free iron inhibits the H^+-ATPase

The H^+-ATPase activity of CRMF was significantly inhibited (80–90%) following exposure to $FeSO_4$ or $FeCl_3$ (Fig. 1). The IC_{50} values found for inhibition were 25 μM and 50 μM for Fe^{2+} and Fe^{3+}, respectively. The ATP-dependent H^+ pumping activity of CRMF vesicles was inhibited in parallel by iron, as indicated by measurements using the H^+ gradient-sensitive fluorescent probe 9-amino-6-chloro-2-methoxyacridine (data not shown). Inhibition of the ATPase was caused by free iron, as the addition of metal ion chelators such as EDTA (not shown), *o*-phenanthroline (Fig. 2) or deferoxamine (not shown) to the reaction medium containing iron completely prevented inhibition.

3.2. Irreversible inhibition of the H^+-ATPase by Fe^{2+}

To investigate whether the inhibition of the ATPase activity could be reversed by iron chelation, 200

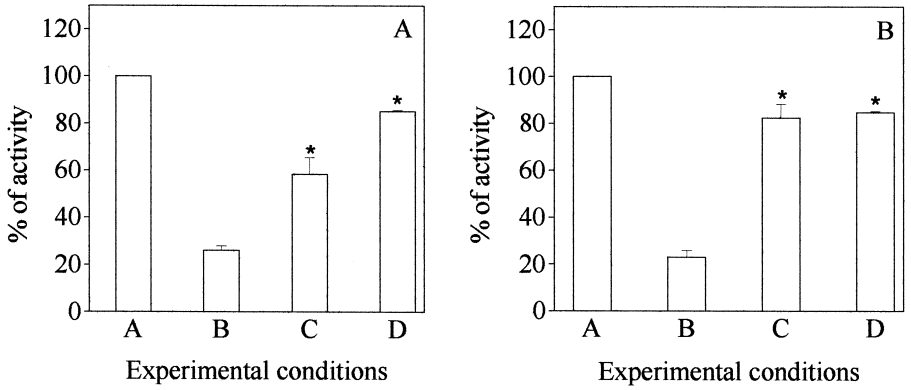

Fig. 3. Inhibition of the ATPase activity is only partially reversed by Fe^{2+} chelation. The ATPase activity was assayed in the presence of 100 μM Fe^{2+} (panel A) or Fe^{3+} (panel B). Columns: A, EDTA (200 μM) was added to the medium from the beginning and the activity was measured for 30 min (non-inhibited condition); B, the activity was measured in the absence of EDTA (inhibited condition); C, EDTA (200 μM) was added to the medium 5 min after starting and the mixture was then further incubated for 25 min to complete a total of 30 min (experimental condition); D, expected activity (see description in Section 3). Bars represent averages ± S.D. from 4–5 experiments. *Significant ($P < 0.05$) when compared with the inhibited condition (column B).

μM EDTA was added to the medium 5 min after initiating the ATPase activity assay in the presence of iron (100 μM). The mixture was then further incubated for 25 min to complete a total of 30 min, and the total amount of P_i released was determined. The samples to which EDTA was added at time zero (i.e., simultaneously with the addition of iron) or after 30 min of incubation in the presence of iron were utilized for calculating the control (non-inhibited) and maximally inhibited activities, respectively. If the inhibition of the ATPase activity were fully reversible upon iron chelation, then the total amount of P_i released in 30 min for any given experimental condition should be proportional to the fractions of time that the enzyme was subjected to each condition (i.e., 5 min inhibited and 25 min non-inhibited). Interestingly, the experimentally measured amounts of P_i released were significantly lower than the expected values when the preincubation was performed in the presence of Fe^{2+} but not in the presence of Fe^{3+} (Fig. 3A,B). This suggests that the inhibition of the H^+-ATPase by Fe^{2+} was partly due to irreversible inactivation of the enzyme.

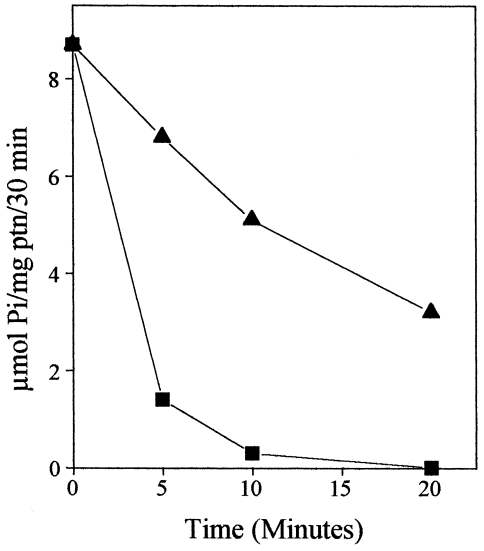

Fig. 4. Irreversible inhibition of H^+-ATPase activity by preincubation with Fe^{2+} (■) or Fe^{3+} (▲) in the absence of ATP. CRMF was preincubated for the indicated amounts of time in the presence of 100 μM iron. The ATPase activity assay was then started by addition of ATP (2 mM) plus EDTA (200 μM). The data represent a typical experiment.

3.3. *ATP prevents iron-induced inactivation of H^+-ATPase activity*

The requirement of ATP for iron-dependent inactivation of the ATPase was investigated by preincubating plasma membrane H^+-ATPase in the presence of 100 μM iron for different times in the absence of ATP. The ATPase activity assay was then started by addition of 2 mM ATP plus 200 μM EDTA (Fig. 4). When compared to the experiment shown in Fig. 3, where ATP was present from the start in the preincubation medium containing iron, significantly stronger inhibition was observed when the membranes

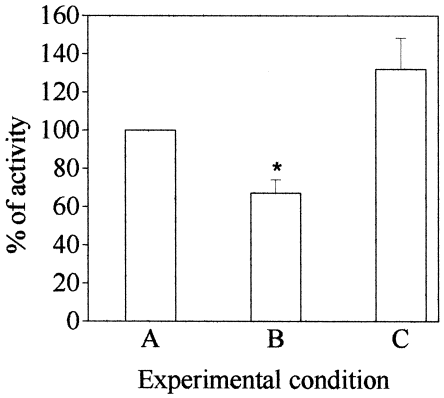

Fig. 5. Protection of ATPase activity by preincubation with ATP. CRMF were preincubated in the absence (A) or in the presence of 100 μM Fe^{2+} (B,C). ATP (2 mM) was either absent (B) or present (C) during preincubation with Fe^{2+}. After 20 min of preincubation, the ATPase activity assay was started by addition of 2 mM ATP and 200 μM EDTA (B) or by 10 mM $MgCl_2$ and 200 μM EDTA (C). Data represent means (±S.D.) of at least three different experiments. *Significant ($P < 0.05$) when compared with column A.

were previously exposed to Fe^{2+} in the absence of ATP for periods as short as 5 min (35% inhibition when ATP was present in the medium (Fig. 3) versus 70% inhibition when ATP was absent (Fig. 4)). This result indicates that inactivation of the H^+-ATPase promoted by iron does not require ATP and that, in fact, the nucleotide significantly protects the enzyme from inactivation.

The plant plasma membrane H^+-ATPase does not hydrolyze ATP in the absence of Mg^{2+}. Under steady state conditions of ATP hydrolysis (i.e., in the presence of Mg^{2+}), the enzyme cycles between different conformational states [18]. In order to test whether the protective effect of ATP was due to stabilization of one of those intermediate conformations in particular, the enzyme was preincubated with 100 μM iron in the absence or in the presence of 2 mM ATP. When ATP was present, the assay was carried out in the absence of Mg^{2+} to avoid initiation of the catalytic cycle. After 20 min of preincubation, 200 μM EDTA was added to chelate iron and the ATPase activity assay was triggered by addition of either 2 mM ATP or 10 mM Mg^{2+}, depending on the preincubation condition. Fig. 5 shows that the ATPase activity was significantly protected by preincubating the CRMF with ATP even in the absence of Mg^{2+}, indicating that protection was not related to a particular intermediate state of the ATPase accumulated during the ATP hydrolysis cycle.

3.4. *Inactivation involves ATPase thiol oxidation by lipoperoxides*

Iron is a well-known catalyst of the formation of ROS, which can oxidize proteins, including P-type ATPases, leading to loss of function [19–21]. Among other possible mechanisms, ROS can damage enzymes by direct oxidation of cysteine residues and/or indirectly through peroxidation of membrane

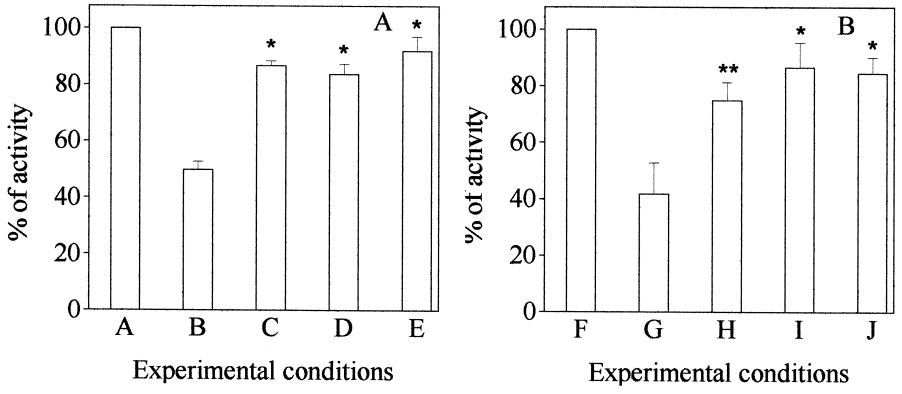

Fig. 6. Effect of DTT and BHT on ATPase inhibition by Fe^{2+}. Crude microsomes (panel A) or sucrose gradient preparation (panel B) were preincubated for 20 min in the absence (A,F) or in the presence (B–E, G–J) of 100 μM Fe^{2+}. The ATPase assay was started by addition of 2 mM ATP plus 200 μM EDTA. A,F, control; B,G, Fe^{2+} alone; C,H, Fe^{2+} plus 5 mM DTT; D,I, Fe^{2+} plus 40 μM BHT; E,J, Fe^{2+} plus DTT and BHT. Data represent means (±S.D.) of at least three different experiments. *Significant ($P < 0.05$), **significant ($P < 0.1$) when compared with column B (panel A) or column G (panel B).

phospholipids [10,22]. Therefore, the possible involvement of ROS in the irreversible inhibition of the H^+-ATPase activity was investigated. In the experiments shown in Fig. 6A, CRMF were preincubated with 100 μM Fe^{2+} for 20 min, in the presence or in the absence of 40 μM butylated hydroxytoluene (BHT; a blocker of lipid peroxidation chain reactions) and/or 5 mM DTT (a thiol reducing agent). The ATPase activity assay was then started by addition of 2 mM ATP and 200 μM EDTA. Nearly complete protection from iron-induced inhibition was achieved in the presence of BHT and DTT (either individually or in combination). A similar protection profile was observed using a further purified preparation of plasma membrane (Fig. 6B). In parallel experiments, the extent of lipid peroxidation was determined by measuring the amount of TBARS released in each condition. A significant TBARS level was verified when the plant microsomal fraction was preincubated in the presence of iron. In contrast, a lower TBARS level, similar to that observed in a control sample preincubated in the absence of iron, was observed when 40 μM BHT was present in the medium (Fig. 7). Taken together, these results suggest that iron does not promote the inactivation of corn root plasma membrane H^+-ATPase by direct thiol oxidation but rather via lipid peroxidation.

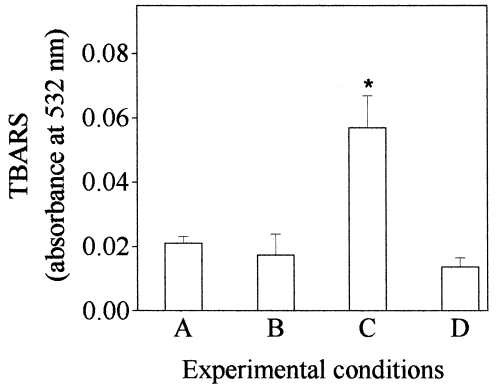

Fig. 7. Formation of TBARS in plasma membrane vesicles in the presence of Fe^{2+}. Vesicles were preincubated for 20 min in the absence (A,C) or in the presence (B,D) of 40 μM of BHT. A,B, without Fe^{2+}; C,D, with 100 μM Fe^{2+}. The reaction was started with addition of 2 mM ATP plus 200 μM EDTA. After 30 min of reaction an aliquot was taken to measure peroxidation of CRMF. Data represent means (±S.D.) of three different experiments. *Significant ($P<0.05$) when compared with column A.

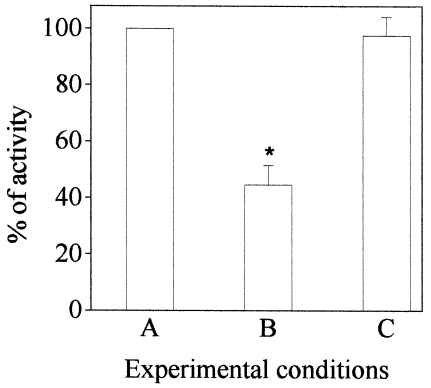

Fig. 8. Effect of hypoxia on the inhibition of H^+-ATPase activity by Fe^{2+}. CRMF were preincubated for 20 min in the absence (A) or in the presence of 100 μM Fe^{2+} (B,C), in an atmosphere of argon (C) or normal atmosphere (A,B). The ATPase activity assay was started by addition of 2 mM ATP. Data represents means (±S.D.) of six different experiments. *Significant ($P<0.05$) when compared with column A.

3.5. Iron-induced inactivation requires O_2

Iron-mediated lipid peroxidation is most frequently observed when H_2O_2 is present in the medium, due to formation of the highly reactive hydroxyl radical ($HO^{\bullet}$) by Fenton chemistry [10]. However, this does not seem to be the case in the experiments presented above, as addition of catalase (up to 100 IU/ml) to the reaction medium did not prevent H^+-ATPase inactivation (not shown). Thus, the participation of dissolved O_2 as a pro-oxidant was investigated. For this purpose, H^+-ATPase activity was assayed in reaction medium deoxygenated by continuous bubbling with a stream of argon, in the absence or in the presence of 100 μM iron. The significant decrease in iron-mediated inhibition in argon-saturated atmosphere (Fig. 8) points to a pivotal role of O_2 in the oxidative damage of the H^+-ATPase.

3.6. Iron-induced inactivation is accompanied by loss of the H^+-ATPase band in SDS–PAGE

Protein oxidation by ROS leads to structural alterations ranging from single group modification to aggregation or fragmentation of the polypeptide chain [19,20,23–25]. Indeed, both protein fragmentation and aggregation have been shown to take place in the oxidative inactivation of the sarcoplasmic reticu-

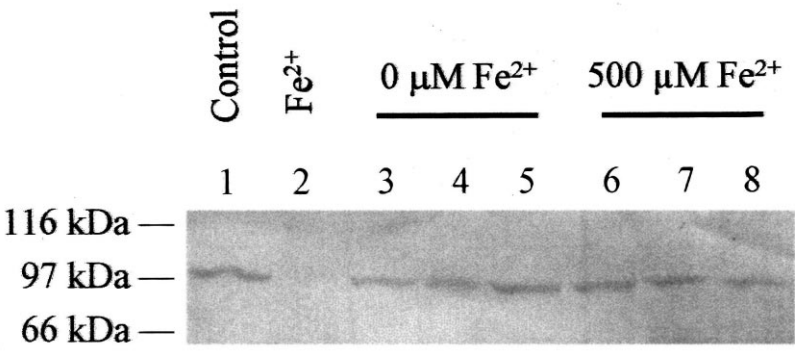

Fig. 9. Western blot analysis of PMA. Lanes: 1, control CRMF; 2, CRMF (200 μg/ml) preincubated for 20 min in the presence of 500 μM Fe^2 (note the disappearance of the ATPase band); 3–5, CRMF incubated in the absence of Fe^{2+} in medium containing 30 mM DTT, 250 μM BHT or DTT plus BHT, respectively; 6–8, CRMF incubated in the presence of 500 μM Fe^{2+} in medium containing 30 mM DTT, 250 μM BHT or DTT plus BHT, respectively. Identical amounts (200 μg) of CRMF total protein were applied in each lane.

lum Ca^{2+}-ATPase by iron [26,27]. A polyclonal antiserum raised against yeast plasma membrane H^+-ATPase was used to identify the plasma membrane H^+-ATPase in Western blots of samples preincubated with iron under different experimental conditions. A 100 kDa protein band compatible with the H^+-ATPase was detected by the antiserum in a control sample not preincubated with iron (Fig. 9, lane 1). However, when the CRMF were preincubated with 500 μM iron for 20 min the ATPase band disappeared (lane 2), unless 250 μM BHT and/or 30 mM DTT were also added to the preincubation medium (lanes 6–8). These results suggest that oxidative damage promoted by iron results in aggregation and/or fragmentation of the plasma membrane H^+-ATPase.

4. Discussion

Because plants are devoid of locomotion, they are inexorably exposed to specific environmental conditions. Thus, their survival depends on effective homeostatic control and efficient transport of nutrients, which are generally present at very low concentrations in the soil. Among such nutrients, heavy metals, known to be essential for many protein functions, must be present in cells at highly regulated levels because overloading may be harmful to the plant. Heavy metals may interact with a number of organic molecules, leading to undesirable modifications of their bioactivity [28]. This is the case of iron, whose concentration in the soil can reach excessive levels either naturally or due to pollution.

Iron has been well characterized as a powerful generator of ROS in many organisms, including plants [29]. For example, iron-induced oxidative stress has been described in *Nicotiniana plumbaginifolia* [30]. Reports of the involvement of ROS in a number of physiological and pathological cellular events are abundant in the literature [10]. Many enzymes are readily inactivated by these free radicals, becoming more susceptible to proteolytic degradation. The accumulation of such 'tagged' proteins has been associated with many pathological processes including cell aging and death [31]. Ionic unbalance has been identified as one the first events leading to cell death, suggesting that ion transport systems may be early targets for oxidative damage [11,32,33]. Indeed, there are many reports in the literature concerning impairment of transport ATPases by interaction with ROS either by direct oxidation of amino acid residues or via peroxidation of membrane phospholipids (for a recent review, see [11]).

Understanding the effects of iron on the plasma membrane of plant roots is relevant not only for basic reasons regarding plant growth in environments with high levels of iron, but also from the perspective of developing seeds with increased amounts of bioavailable iron, which could be a good substitute for meat as a less expensive dietary source of this metal [34].

In the present study, we describe the iron-depen-

dent irreversible inactivation of corn root plasma membrane H^+-ATPase. Several lines of evidence indicate that ROS are involved in the inactivation mechanism. First, BHT prevented both lipid peroxidation and loss of ATPase activity when added together with Fe^{2+} to the medium. Second, protection against inactivation could be achieved by decreasing the concentration of oxygen in the medium. Third, oxidation of protein sulfhydryl groups must be involved, as DTT was also effective in protecting against iron-induced inactivation.

The fact that blockage of lipid peroxidation was able to prevent the inhibition of ATPase activity indicates that protein thiols were not the primary targets of oxidation by iron-generated ROS. Rather, thiol groups were likely secondarily oxidized by lipoperoxides. Thiol oxidation might lead to the formation of intermolecular disulfide bridges, leading to formation of high molecular weight aggregates of the H^+-ATPase and explaining the disappearance of the H^+-ATPase band in the Western blot experiments when CRMF were incubated with Fe^{2+} in the absence of either BHT or DTT. In addition to the H^+-ATPase band, other protein bands were also found to disappear from stained SDS–PAGE gels of both crude and sucrose gradient purified membrane preparations following exposure to Fe^{2+} (data not shown). We have also observed that addition of 30 mM DTT to these samples increased indiscriminately the band intensities. This result was expected as any protein in the membrane can be a target for free radicals or lipoperoxides and/or aggregate upon thiol oxidation. Aggregation of oxidatively damaged proteins via disulfide bridge formation has been widely reported in the literature (for example, see [35] and references therein). Under our SDS–PAGE conditions, the concentration and redox potential of β-mercaptoethanol present in the sample loading buffer were not sufficient to dissociate cross-linked proteins. This has also been observed in a previous paper dealing with oxidative damage of the sarcoplasmic reticulum Ca^{2+}-ATPase by iron, where the aggregates could only be dissolved by addition of high DTT concentrations [26].

Alternatively, iron/ROS-induced protein fragmentation may also occur, as previously reported for the F_1-ATPase [36] and the sarcoplasmic reticulum Ca^{2+}-ATPase [26,27]. In the latter case, it has been proposed that oxidative damage was due to generation of ROS by iron bound to specific site(s) in the ATPase, via metal catalyzed oxidation (MCO), as previously described by Stadtman for other systems [37]. In this type of mechanism, ROS are generated in situ, at the site of iron binding to the protein, and react with neighboring amino acid residues. Thus, MCO reactions are not usually sensitive to the presence of water soluble scavengers for ROS. By contrast, protection against oxidation may be achieved in the presence of specific protein ligands that affect iron binding to the protein. In the inactivation of the H^+-ATPase, we have observed two of the criteria normally used to characterize MCO, i.e., the lack of protection by mannitol (a very effective water soluble scavenger against hydroxyl radicals; data not shown) and protection by ATP (a natural protein ligand). However, the following observations argue against an MCO mechanism in this case. Protection against ROS-induced oxidative damage by ATP binding to the catalytic site has been described for other P-type ATPases [38,39], suggesting that oxidation may involve residues located in the vicinity of the active site. Indeed, exposure of the sarcoplasmic reticulum Ca^{2+}-ATPase to different pro-oxidants, such as peroxynitrite [40], 2,2′-azobis(2-amidinopropane) dihydrochloride or hydrogen peroxide [41] resulted in modification of amino acids located in the nucleotide binding site. In the first case, reversible disulfide bridges were formed and the authors suggested a regulatory role for peroxynitrite in the normal cellular function of the calcium pump. In the latter case, the Ca^2+-ATPase monomers were linked by bi-tyrosine cross-links between each of the nucleotide binding domains. Protection by ATP, however, is not necessarily related to competition with iron for binding to the enzyme, as substrate-induced changes in the exposure and/or reactivity of thiols have been early demonstrated for this enzyme [42]. In addition, protection by ATP could also be explained by iron chelation by the negatively charged phosphates of the nucleotide.

Moreover, as lipid peroxidation appears to be an essential step for corn root plasma H^+-ATPase inactivation (Figs. 6 and 7), it seems more likely that upon iron binding to CRMF phospholipids, or in their neighborhood, the lipoperoxides so formed could easily diffuse and interact with membrane

bound proteins, including the H^+-ATPase, promoting oxidation of thiol groups.

Finally, generation of ROS in the present work does not seem to be related to Fenton chemistry ($Fe^{2+}+H_2O_2 \rightarrow {}^{\bullet}OH+OH^-+Fe^{3+}$), as addition of catalase or superoxide dismutase to the medium did not prevent oxidation (data not shown). This eliminates the participation of H_2O_2 or O_2^- as the oxidant species. In fact, it has been proposed that the initiation of iron-dependent oxidative reactions in membranes may be attributed to radicals other than hydroxyl. The ferryl radical does not appear to be a likely candidate, as H_2O_2 is required for its formation ($[Fe^{2+}+H_2O_2 \rightarrow FeOH^{3+}$ (or FeO^{2+})$+OH^-]$) [10]. On the other hand, the perferryl radical (generated by the reaction: $[Fe^{2+}+O_2 \leftrightarrow (Fe^{2+}\text{-}O_2 \leftrightarrow Fe^{3+}\text{-}O_2^-) \leftrightarrow Fe^{3+}+O_2^-]$) is the most probable candidate, despite its poor reactivity to abduct hydrogen from or to append O_2 to polyunsaturated fat acids. In fact, kinetic studies of lipid peroxidation have shown that the early events in the chain reaction of ROS formation in microsomes and liposomes require a $Fe^{2+}/Fe^{3+}/O_2$ complex or at least a critical ratio of Fe^{2+}/Fe^{3+} [10,43,44], indicating a complex chemistry for this phenomenon.

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