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Biochimica et Biophysica Acta 1414 (1998) 175–187

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Comparison of plasma membrane H^+-ATPase activity in vesicles obtained from dry and hydrated maize embryos

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Received 4 June 1998; received in revised form 26 August 1998; accepted 26 August 1998

Abstract

ATP hydrolysis from H^+-ATPase of plasma membrane was measured in vesicles from maize embryos imbibed at times between 0 and 5 h. The activity had a maximum at 2 h of imbibition. In order to detect whether the enzyme had the same characteristics through the first 5 h of imbibition, vanadate and lysophophatydilcholine sensitivities, as well as trypsin, pH and temperature effects on the activity of the H^+-ATPase from plasma membrane vesicles isolated from embryos imbibed at 0 or 5 h were studied. The results indicate that the activity expressed at 0 h is very different from the activity at 5 h. The activity from embryos imbibed for 5 h was less sensitive to vanadate, trypsin and lysophosphatidylcholine, more sensitive to denaturing temperatures and with a broader pH dependence, as compared to the activity from embryos that were not imbibed. When vanadate-sensitive ATPase activity was purified by anion exchange chromatography, the peaks obtained from the 0 and 5 h imbibed embryos were different and non-overlapping. These data could be interpreted in terms of different enzyme structures from dry and imbibed embryos due to either different primary structures or covalent modifications, or differences in membrane vicinities.

Keywords: Embryo germination; H^+-ATPase; Plant plasma membrane ATPase; Plasma membrane; Seed germination; *Zea mays*

1. Introduction

When a seed is imbibed, structural and metabolic changes occur in the embryo before radicle protrusion, which is the culmination of seed germination [1,2]. Many of these changes involve cellular membranes. It has been reported that the lipid bilayer undergoes structural modifications as phase transitions, as a consequence of the passage of the seed from the dry to the hydrated state [3–7]. However, information on the activity of membrane proteins in the transition from the dry to the hydrated state is scarce. Some of these enzymes are required to support growth in more advanced stages of seed germination, in order to produce radicle protrusion. The H^+-ATPase, a housekeeping enzyme, is one of the

Abbreviations: AHA, *Arabidopsis* H^+-ATPase; Btp, 1,3-bis-[Tris(hydroxymethyl)-methylamino]propane; $C_{14}E_8$, polyoxyethylene-8-myristyl ether; CCCP, carbonylcyanide *m*-chlorophenyl-hydrazone; DTT, dithiothreitol; LPC, lysophosphatidylcholine; MES, 2(*N*-morpholino)ethanesulfonic acid; MOPS, 3(*N*-morpholino)propane-sulfonic acid; PEG, polyethyleneglycol; TLCK, Na-*p*-tosyl-L-lysine chloro-methyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloro-methyl ketone

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0005-2736/98/$ – see front matter
PII: S0005-2736(98)00164-3

plasma membrane enzymes that can fulfill some physiological functions associated to germination.

The H^+-ATPase from the plant plasma membrane is an amphipathic protein that translocates H^+ against a concentration gradient: from the cytosol (0.1 μM H_3O^+) to the apoplastic space (about 10 μM H_3O^+), at the expense of energy derived from ATP hydrolysis [8–10]. The electrochemical H^+ gradient thus generated can be used for secondary transport [11–14], cell elongation [15,16], and pH regulation [17–19]. Previous work showed that this enzyme is present and potentially active in embryos from dry maize seeds and that the extent of activity changed in embryos imbibed for 1 and 7 h [20]. The present work describes the dynamics of the activity of the plasma membrane H^+-ATPase and the different properties that it displays during the first 5 h of imbibition, when water penetrates the dry maize embryo, and no radicle elongation is still visible.

2. Materials and methods

2.1. Biological material

Embryos were dissected from mature, dry maize seeds (*Zea mays* L. hybrid Montecillos $A_6 0_2$, Colegio de Posgraduados, Montecillo, Edo. de Mexico) using a scalpel and removing the endosperm. The integrity of the embryos was reflected in a germination of 96%, the same as that reached by the whole kernels and which coincided with the Trypan blue exclusion test [21].

2.2. Germination tests

Embryos were sterilized with 0.12% sodium hypochloride for 2 min, washed twice with distilled water, placed on wet filter paper in Petri dishes, and incubated at 29°C in darkness for different times. For germination recording, 30 embryos were imbibed from 0 to 72 h. The embryos were considered to be germinated when the radicle reached at least 1 mm of elongation.

2.3. Water uptake

Three hundred and ninety sterilized embryos were placed in 13 Petri dishes and samples of 30 embryos were taken to determine fresh weight at the indicated times.

2.4. Isolation of plasma membrane vesicles

At the end of each incubation time (0, 1, 2, 3 or 5 h), batches of 200 embryos were immediately frozen with liquid N_2, ground initially with mortar and pestle, and then with a Tissue Tearor (Biospec products, Oklahoma, USA) at 12000 rpm for 4 min to produce a fine powder. The powder was suspended in homogenization buffer containing 250 mM sucrose, 2 mM EDTA, 1 mM ATP, 70 mM Tris-HCl pH 8.0, 15 mM β-mercaptoethanol, 4 mM dithiothreitol (DTT), 100 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 50 μg/ml Na-*p*-tosyl-L-lysine chloro-methyl ketone (TLCK) and 7 μg/ml chymostatine, at a ratio of 1.5 ml buffer per g of embryo fresh weight. The homogenate was filtered through four layers of cheesecloth and centrifuged at 1000 g for 7 min. The supernatant was used to obtain an enriched plasma membrane vesicles by the aqueous PEG/Dextran two-phase partitioning system [22], following the modifications described by Sánchez-Nieto et al. [20].

2.5. Solubilization and purification of the enzyme

The procedure was performed according to Johansson et al. [23] with some modifications. Plasma membranes at a concentration of 4 mg/ml were mixed with an equal volume of buffer A containing 10 mM MOPS/BTP pH 7.0, 20% (v/v) glycerol, 5 mM EDTA, 0.1 mM DTT, 0.5 mM ATP and supplemented with 1 M KCl and 1.5 mg/ml $C_{14}E_8$. This buffer was added dropwise under continuous stirring and followed by an incubation at room temperature for 30 min. The mixture was then centrifuged at 100000×*g* for 45 min. The pellet was resuspended in the initial volume of buffer A and the enzyme was solubilized by dropwise addition of buffer A containing 20 mg/ml dodecyl-β-D-maltoside (1 ml resuspended pellet per 1 ml buffer for solubilization; for optimal recovery of the ATPase activity the detergent/protein ratio was about 10), under continuous stirring. After 30 min at room temperature, unsolubilized material was pelleted at 100000×*g* for 45

min. The supernatant was applied to a MonoQ HR5/5 (Pharmacia) FPLC anion-exchange column. The column was equilibrated with 10 mM histidine-HCl, 5% (w/v) glycerol, 0.1 mM EDTA, 0.5 mM ATP, pH 6. Bound protein was eluted by applying a 0–1.0 M linear gradient of NaCl at a flow rate of 0.3 ml/min. Fractions of 0.5 ml were collected and assayed for ATPase activity in the presence and absence of sodium orthovanadate.

2.6. Lipid activation of the H^+-ATPase

Soybean phospholipids were added to restore the ATPase activity lost during the purification of the H^+-ATPase [23]. A solution consisting of 10 mM MOPS/Btp pH 7.0, 5% (v/v) glycerol, 0.1 mM DTT, 2 mg/ml asolectin and 2 mg/ml dodecyl-β-D-maltoside was mixed with an equal volume of the column eluate or the supernatant of the dodecyl-β-D-maltoside solubilization, and the mixture was incubated for 8 min at room temperature before doing ATPase activity measurements.

2.7. Determination of ATPase activity

ATP hydrolysis from plasma membrane H^+-ATPase (EC 3.6.1.35) was measured as described in Sánchez-Nieto et al. [24] in 150 μl of assay medium that contained: 250 mM sucrose, 7 μM carbonylcyanide *m*-chlorophenyl-hydrazone (CCCP) and 2 mM sodium azide to prevent ATP hydrolysis from traces of contaminant mitochondrial ATPase (EC 3.6.1.3). ATP/Tris pH 7.0 and $MgCl_2$ were added at the concentration indicated in each experiment. The calculation of the concentration of ATP and Mg^{2+} complexes was done as described in Rodríguez-Sotres and Muñoz-Clares [25] and the substrate concentration was given in terms of ATPHMg [24]. The standard reaction mixtures also included 100 μM (54 μg/ml) egg lysophosphatidylcholine (type I, Sigma), or concentrations between 15 and 125 μg/ml in titration experiments. Brij 58 had no effect on ATPase activity, either in 0 or 5 h preparations. Therefore, this detergent was not added in further experiments. Sodium orthovanadate prepared according to Gallagher and Leonard [26], was used in concentrations from 10 to 300 μM. ATP hydrolysis was initiated by the addition of 3.3 μg of membrane protein. After 3 h at 30° C, the reaction was stopped by adding SDS (12% final concentration). Released Pi was determined with the method of González-Romo et al. [27]. All assays were done in triplicate for each treatment and all experiments were repeated with three to six different membrane preparations, each one at least twice.

Data from ATP hydrolysis in the presence of inhibitor were analyzed with the aid of non-linear regression algorithms implemented in the software package Origin (Microcal Software, Northampton, MA, USA) by fitting the experimental points to the following equation:

$$v = V_0\, I_{50}/(I_{50} + [VO_4^{3-}]) \quad (1)$$

where, V_0 is the activity in the absence of inhibitor and v the observed activity.

2.8. Determination of ITPase activity

Ca^{2+}-dependent ITP hydrolysis was determined in a medium containing 250 mM sucrose, 20 mM MOPS/Btp pH 7.0, 2 mM NaN_3, 7 μM CCCP, 10 mM ITP, 10 mM $MgCl_2$, 100 mM EGTA, 54 μg/ml LPC and 50 μM free Ca^{2+}, which was estimated as described by Carnelli et al. [28]. The reaction was started by addition of 3.3 μg of plasma membrane protein and stopped by 24% SDS addition. The released phosphate was determined as described by González-Romo et al. [27].

2.9. Trypsin treatment

A 600 μg amount of plasma membrane protein was added to 480 ml of proteolysis medium containing 250 mM sucrose, 2 mM DTT, 5 mM EDTA/Btp pH 7.5 and 4 mM ATP/Btp pH 7.5. This was mixed with an equal volume of proteolysis medium supplemented with 24 μg of soybean trypsin (Boehringer Mannheim). After 20 min of digestion at 20°C, the reaction was stopped with the addition of 240 μg of soybean trypsin inhibitor in 240 μl of proteolysis medium. The membranes were washed with 5 vols. of solution containing 250 mM sucrose and 20 mM MOPS/Btp pH 7.0. After centrifugation at $100\,000 \times g$, the pellet was resuspended in the same solution at a concentration of about 1.0 mg/ml. Measurements of ATP hydrolysis were immediately performed.

2.10. pH dependence of ATPase activity

ATP hydrolysis was measured in the assay mixture described above (with 8.11 mM ATPHMg and 35 μM free Mg^{2+}), but buffered with 50 mM MES/Btp when pH was ≤7.0 or using 50 mM MOPS/Btp when pH was ≥7.0. Activity at pH 7.0 was the same regardless of the buffer employed. The calculation of the parameters V_{max}, pK_1, and pK_2 were performed according to Segel [29], or by fitting the above data to the Eq. 2:

$$V = V_{max}/(1 + 10^{(pK_1 - pH)} + 10^{(pH - pK_2)}) \quad (2)$$

2.11. Effect of denaturing temperatures on ATPase activity

A 3.3 μg amount of plasma membrane vesicles in an ATPase assay mixture without ATP and $MgCl_2$, was incubated at 35, 40, 45, 50, 55 or 60°C, in a water bath for 10 min, and then the tubes were immediately transferred to a water bath at the temperature of the ATPase assay (30°C). The activity of the latter samples was determined by mixing with ATP and $MgCl_2$ (8.11 mM ATPHMg final complex concentration and 35 μM free Mg^{2+}), the reaction was arrested after 3 h and the remaining enzyme activity was measured as described above.

2.12. Protein determination

The method of Peterson [30] with bovine serum albumin as standard was used.

3. Results

3.1. Water uptake and germination

When dissected embryos were imbibed in water, a rapid increase in their fresh weight took place (phase I) followed by a slower phase (phase II) and finally by a faster phase of fresh weight increase (phase III, Fig. 1), resembling the pattern previously observed in whole seeds [2]. The first phase of water uptake has been described as a period in which some tissue injury takes place, and the second to the time when a re-assumption of metabolic activity and re-organization of cell structures occur [2]. Fig. 1 shows that in our cultivar, phase II has already started after 1 h of imbibition and phase III was initiated at about 8 h. The latter was close to the time when radicle elonga-

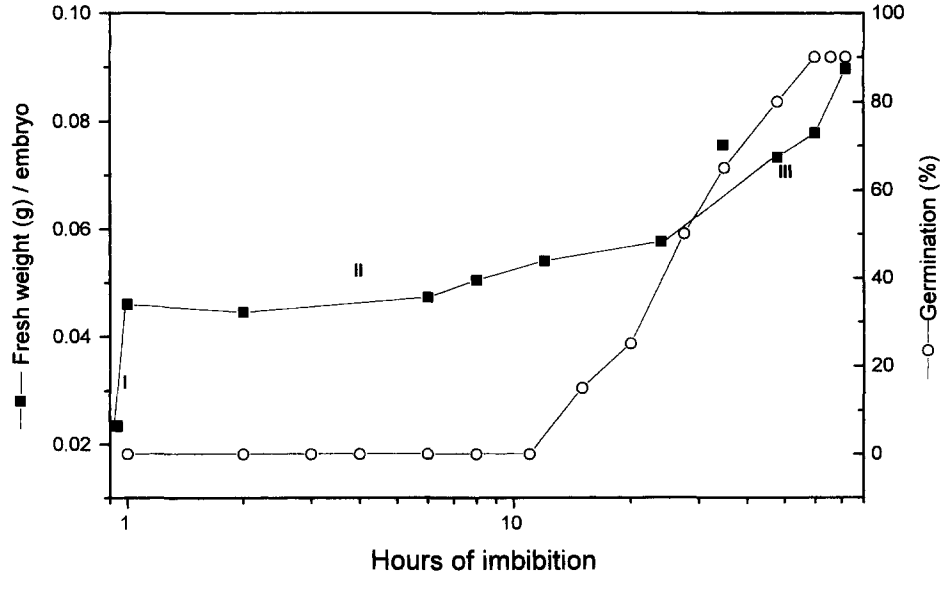

Fig. 1. Time course of germination (○) and fresh weight (■) increase of maize embryos. Embryos were imbibed at the indicated times and batches of 30 embryos were used to measure viability and fresh weight. This experiment is representative of three different experiments.

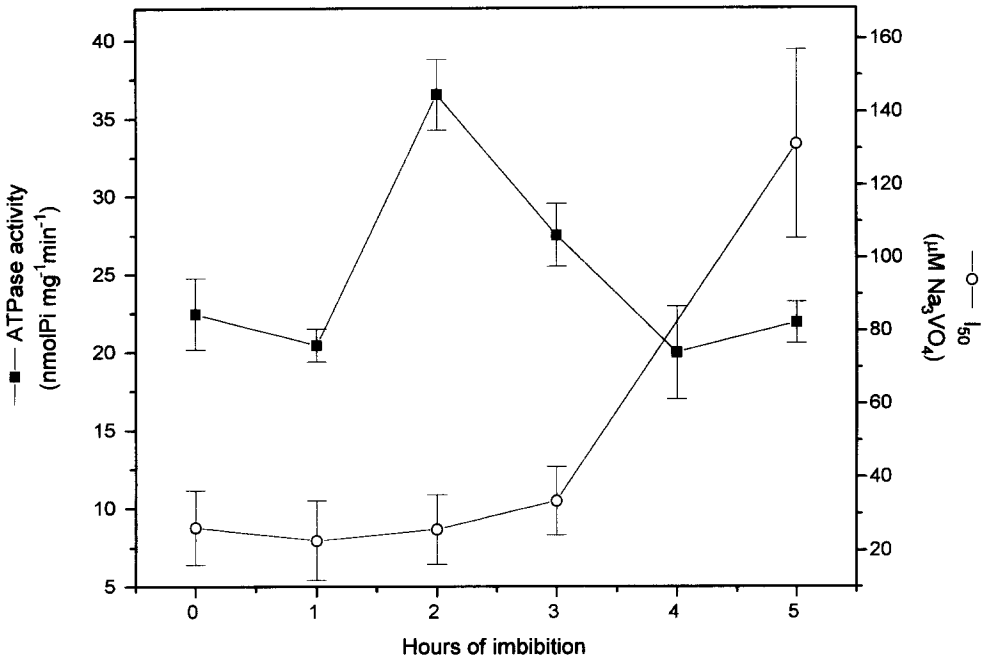

Fig. 2. Patterns of ATPase activity and vanadate sensitivity at early imbibition times. ATPase activity (■): 200 g of embryos were imbibed for the indicated times, then were homogenized in liquid N_2. The plasma membrane enriched fraction was obtained by the aqueous polymer two-phase partitioning system and the resulting U_2 fraction was used to measure ATP hydrolysis with 3.3 μg of membrane protein. The assay medium consisted of 250 mM sucrose, 20 mM Tris-HCl pH 7.0, 2 mM NaN_3, 100 μM (54 μg/ml) lysophosphatidylcholine, 8.11 mM ATPHMg and 35 μM free Mg^{2+}. Vanadate sensitivity of ATPase activity (○): the inhibition of plasma membrane H^+-ATPase activity by increasing concentrations of Na_3VO_4 was determined in plasma membrane vesicles obtained from 0, 1, 2, 3 and 5 h imbibed embryos. To obtain the I_{50} values, increasing vanadate concentrations were added to the reaction medium and the vanadate-sensitive ATPase activity was fitted to Eq. 1 in Section 2.

tion started (as inferred from the germination percent which was about 15% at 15 h of imbibition). The germination curve (Fig. 1), shows that during the first 10 h of imbibition, no germination of embryos took place.

We followed the activity of the plasma membrane H^+-ATPase during the first 5 h of imbibition; this included phase I and the initial part of phase II, when the metabolism reactivation starts to take place and there is no germination yet, assessed as radicle protrusion [2].

3.2. ATPase activity profile

The activity of the plasma membrane H^+-ATPase was measured as vanadate-sensitive ATP hydrolysis in plasma membrane vesicles purified from embryos imbibed from 0 to 5 h (Fig. 2). The non-imbibed embryos showed a high activity that increased to a maximum after imbibition for 2 h; this subsequently decreased to a level of constant activity similar to that of the non-imbibed embryos.

3.3. Properties of the enzyme throughout the first hours of imbibition

3.3.1. Vanadate inhibition

To explore if the variation in ATPase activity during the first 5 h of imbibition was associated to changes in some properties of the enzyme, we compared the sensitivity to vanadate, a highly specific inhibitor of the plasma membrane H^+-ATPase [8], on the activity present in membranes isolated from non-imbibed embryos or imbibed for 1, 2, 3 and 5 h. Fig. 2 shows that the enzyme activity from membranes of non-imbibed embryos and those imbibed for 3 h was more sensitive to vanadate than that of membranes after 5 h of imbibition, as it can be deduced from the I_{50} values obtained for vanadate effect. It is shown that the I_{50} values for vanadate

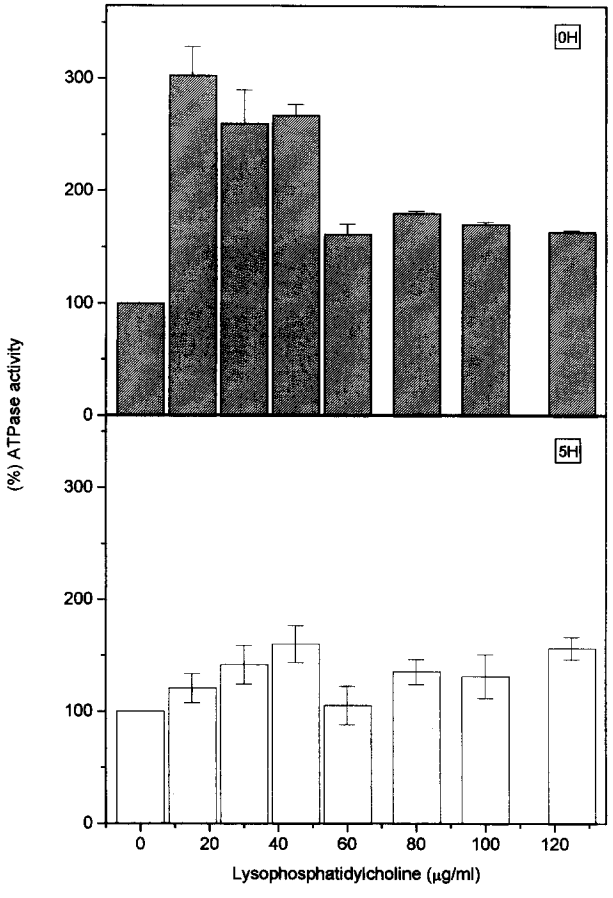

Fig. 3. Effect of increasing lysophosphatidylcholine concentrations in the plasma membrane ATPase activity from embryos imbibed for 0 (upper panel) and 5 h (lower panel). A 3.3 μg amount of membrane protein from 0 and 5 h imbibed embryos was added to an ATPase assay containing 250 mM sucrose, 20 mM Tris-HCl pH 7.0, 2 mM NaN_3, 8.11 mM ATPHMg and 35 μM free Mg^{2+} and lysophosphatidylcholine at the indicated concentrations. Each value was expressed as percent of the activity without lysophosphatidylcholine (100%). The control activities were 13.61 nmol Pi/mg/min and 16.0 nmol Pi/mg/min for 0 and 5 h, respectively.

inhibition of the plasma membrane ATPase activity from embryos imbibed for 5 h was about 5-fold higher than that at earlier times.

In view of the differential sensitivity of ATPase activity to vanadate at early and late imbibition times, we investigated the possible changes in other properties of the enzyme in embryos from an initial time, i.e. 0 h (dry, non-imbibed embryos) and in embryos at longer times, i.e. 5 h (hydrated, imbibed embryos). The peak of activity at 2 h was not further studied.

3.3.2. *Lysophosphatidylcholine activation*

Fig. 3 shows the effect of a plasma membrane H^+-ATPase activator lysophosphatidylcholine (LPC) [31–33] on the ATPase activity of plasma membranes from 0 and 5 h imbibed embryos and we found significant differences. The concentration of LPC required to attain the maximal stimulation of H^+-ATPase activity from non-imbibed embryos was 1.5 times higher than in embryos imbibed 5 h. The activity from non-imbibed embryos exhibited a maximal stimulation 1.35-fold higher, as compared to the activity of embryos imbibed 5 h. The lack of effect of Brij-58 on ATPase activity from both preparations implies that detergent action of LPC can be ruled out (see Section 2).

3.3.3. *pH profile*

The pH profile of the activity from the plasma membrane H^+-ATPase was also different in embryos imbibed for 0 or 5 h. Fig. 4 shows that although both activities exhibited broad peaks, the enzyme from non-imbibed embryos showed larger activity changes in response to the variation of pH. The activity of 0 h was 2.3-fold higher than the activity from 5 h imbibed embryos. Estimates of the optimal pH for both types of embryos were of 5.9 and 6.2 for the 0 and 5 h imbibed embryos, respectively, and the corresponding pK values were 5.5 and 6.3 for dry embryos and of 5.2 and 7.2 for 5 h imbibed ones.

3.3.4. *Temperature stability*

Since enzyme stability at denaturing temperatures is a good parameter to detect differences in protein structure, we measured the behavior of the plasma membrane H^+-ATPase from 0 and 5 h imbibed embryos as function of temperature (Fig. 5). The enzyme from non-imbibed embryos was stable and active up to 40°C for 10 min, but it progressively lost activity as the temperature was raised. In contrast, the activity from 5 h imbibed embryos suffered progressive loss of activity as temperature was raised above 35°C.

3.3.5. *Trypsin sensitivity*

Palmgren et al. [34,35] have shown that cleavage of the C-terminal of the PM H^+-ATPase with trypsin increases the enzyme activity. In order to test if the 0 and 5 h enzymes showed a different response to tryp-

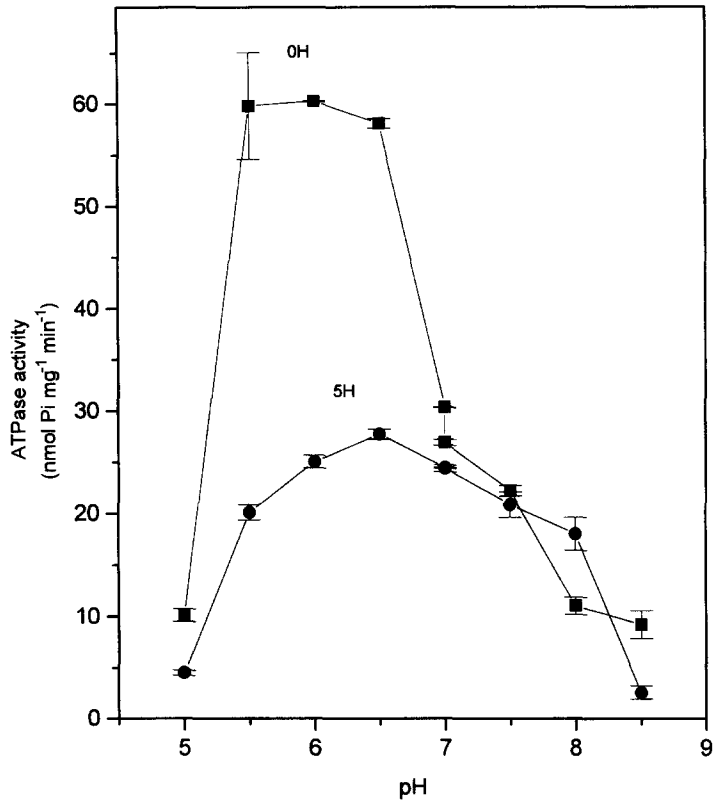

Fig. 4. pH dependence of the plasma membrane ATPase activity from 0 (■) and 5 (●) h imbibed embryos. ATP hydrolysis was assayed in a medium consisting of 250 mM sucrose, 2 mM NaN_3, 100 μM (54 μg/ml) lysophosphatidylcholine, 8.11 mM ATPHMg, 35 μM free Mg^{2+} and 50 mM MOPS/Btp (pH 7.0) or 50 mM MES/Btp (pH 7.0) over the pH range 5.5–8.5. The experimental values were used to obtain the p*K* constants values with Eq. 1 in Section 2.

sin activation, the experiment presented in Table 1 was performed. The results show that the 0 h preparation was stimulated 90% by trypsin and 75% by LPC. On the other hand, the 5 h preparation was stimulated 35% by trypsin and 43% by LPC. The stimulations observed with trypsin and LPC were not additive.

3.4. Purification of the plasma membrane ATPase

A possible explanation for the biochemical differences observed was that non-imbibed embryos possessed one form of the enzyme which was different from the form present in 5 h imbibed embryos. Thus, the plasma membrane H^+-ATPase from membrane preparations of embryos imbibed 0 and 5 h was solubilized with dodecyl-β-D-maltoside and then purified in a FPLC system with an anion exchanger. The yields of the different steps are in Table 2. It is shown that in relative terms, the wash with the detergent $C_{14}E_8$ rendered pellets with similar enrichment in specific activities (1.5-fold and 1.9-fold for 0 and 5 h, respectively) to the preparation obtained in the Johansson et al. procedure (1.8-fold, [23]). In the same way, the solubilized enzyme after dodecyl-β-D-maltoside treatment presented recoveries of specific activity of 1.8-fold and 3.0-fold for 0 and 5 h, respectively, which is in the same range (2.6-fold), as for the analogous enzyme reported by Johansson et al. [23]. The final step of purification produced an 0 h enzyme of a 2.8-fold purification and a 5 h enzyme with a 5.2-fold increase in specific activity. This purification is higher than the one attained by Grandmougin-Ferjani et al. [36], but lower than Johansson et al. procedure [23], which yields a preparation with an 8.6-fold increase in specific activity. It is clear that 0 and 5 h preparations have low specific activities throughout the purification procedure as compared

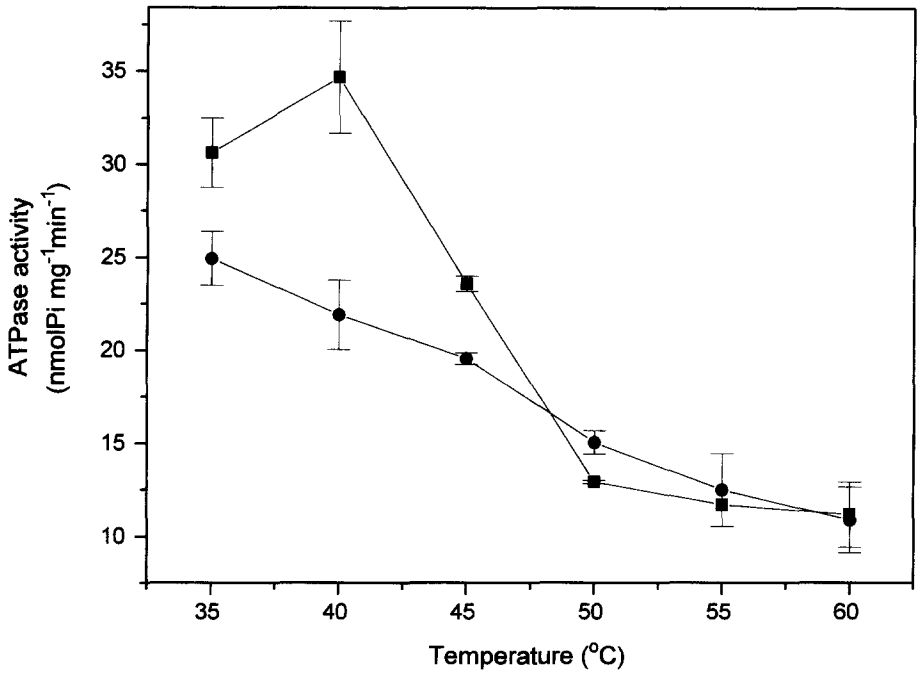

Fig. 5. Sensitivity of plasma membrane ATPase activity to high temperatures. A 3.3 μg amount of membrane protein from plasma membrane vesicles 0 (■) and 5 (●) h imbibed embryos was preincubated in the ATP assay mixture containing 250 mM sucrose, 20 mM Tris-HCl pH 7.0, 2 mM NaN_3 and 100 μM (54 μg/ml) lysophosphatidylcholine for 10 min at the indicated temperature. Then, the mixture was transferred to an incubation temperature of 30°C to measure ATPase activity. This was initiated by the addition of ATP and Mg^{2+} at the final concentration of 8.11 mM ATPHMg and 35 μM free Mg^{2+}.

to other preparations from more conventional sources [23]. This might be due to the nature of tissue, which is embryonic tissue in a quiescent state (0 h) or just about re-starting metabolic functions (5 h).

The MonoQ column elution profiles of the solubilized ATPase from plasma membranes of embryos imbibed for 0 and 5 h are shown in Fig. 6. Each preparation had a unique symmetrical and non-overlapping peak of vanadate-sensitive ATPase activity, but with different retention times. The maximal activity of the peak from dry embryos was found in fraction 12 and that from the imbibed embryos was in fraction 20.

Table 1
Effect of trypsin treatment on the ATPase activity from plasma membrane H^+-ATPase from embryos imbibed 0 and 5 h

Treatment	ATP hydrolysis (nmol Pi/mg/min)	
	0 h	5 h
Control	13.0 ± 0.7	16.1 ± 0.4
Trypsin	24.6 ± 0.7	21.6 ± 0.4
LPC	22.7 ± 0.3	22.9 ± 0.3
Trypsin, LPC	22.9 ± 0.7	22.0 ± 0.6

Plasma membrane vesicles were exposed to trypsin or LPC action as described in Section 2 and after washing. ATPase activity was measured as Pi release.

4. Discussion

The rapid initial phase of water uptake is accompanied by a leakage of solutes from the seed or the embryo [2]. This has been interpreted as an increase of permeability by injury of the cell membranes that simultaneously undergo a process of self-reassembly upon the water influx to the seed tissues [3,6,37]. This phenomenon continues during the subsequent slower phase of water uptake (phase II), when many metabolic pathways are re-established [2,5]. In our study, maize embryos after the first 5 h of imbibition were in the second phase of water uptake. Therefore, we considered that this was an appropriate period of time to study the onset of expression and the course of the H^+-ATPase activity from the plasma membrane upon hydration of the embryo.

The plasma membrane preparations used were

highly pure, as estimated by the enrichment of glucan synthase II (EC 2.4.1.12), a plasma membrane marker enzyme [20]. In addition, similar extents of vanadate-sensitive ATPase activity (about 85%) and the contaminant ATPase (mitochondrial ATPase, about 15%) were recovered in preparations either from non-imbibed or imbibed embryos (results not shown), thus allowing a fair comparison of the activity throughout the course of imbibition.

The ATPase activity of the plasma membrane vesicles obtained from non-imbibed embryos was remarkably significant considering that the tissue was in physiological latency and it was not exposed to water before homogenization. This suggests that at least part of the enzyme synthesized during embryogenesis remains in the membrane and is potentially active through the desiccation period, being able of functioning during early imbibition. This finding is consistent with reports that indicate that cytochrome oxidase (EC 1.9.3.1), ATPase and other enzyme activities from oxidative phosphorylation are present in mitochondria from dry sunflower seeds [38], and maize embryos of dry seeds [39]. It is possible that the plasma membrane H^+-ATPase is related to transmembrane solute transport, an essential function for the survival of the embryo during germination [1]. In fact, because of its importance as a primary pump, this ATPase has been postulated as a housekeeping enzyme [40].

Another characteristic of the enzyme activity profile is the presence of a maximum activity at 2 h of imbibition. There are enzymes as superoxide dismutase (EC 1.15.1.1) [41], peroxidase (EC 1.11.1.17) [41], and mitochondrial ATPase [39], that also show a peak of activity during the first 5 h of imbibition. However, no explanation has been found for this transient increase. In our case, once that the maximum activity was reached, a progressive decrease leading to the same initial levels of activity was observed. However, it is likely that the ATPase activity increases again during radicle elongation, since a direct correlation between cell elongation and ATPase activity in growing coleoptiles has been reported [15].

The changing profile of ATPase activity during the first 5 h of imbibition could be due to several causes: (a) changes of turnover rates, which could involve changes in the amount and/or expression of different isoforms; (b) modification of the enzyme: (c) changes in membrane properties; (e) a combination of more than one of these possibilities. Some of these various alternatives may be the result of differences in structural features of the enzyme.

In relation to the first possibility, the only work

Table 2
Purification of the plasma membrane H^+-ATPase from dry and 5 h imbibed maize embryos by solubilization with dodecyl-β-D-maltoside and by anion exchange chromatography according to Johannsson et al. [23]

Fraction	Protein (mg)		Total ATPase activity (nmol Pi/min)		Specific ATPase activity (nmol Pi/mg/min)	
	0 h	5 h	0 h	5 h	0 h	5 h
Plasma membrane fraction	5.0 (100%)[a]	8.0 (100%)	110.5	174.4	22.1	21.8
$C_{14}E_8$ wash						
Pellet (ATPase)	1.9 (38.0)	4.08 (51)	63.6	169.3	33.5	41.5
Supernatant	3.0	2.95	9.9	17.6	3.3	5.9
Dodecyl-β-D-maltoside solubilization						
Pellet	0.7	1.2	7.77	11.7	11.1	9.8
Supernatant (solubilized ATPase)	1.0 (20)	2.7 (33.7)	40.6	173.3	40.6	64.2
MonoQ fractionation						
Best fraction[b]	0.09 (1.8)	0.08 (1.0)	5.6	9.1	61.9	114.3

Plasma membrane vesicles used for purification were obtained from 20 g of embryos by the aqueous phase partitioning system according to the method of Sánchez-Nieto et al. [20].

[a]Percent of a recovery of each fraction.

[b]Referred as the eluted fraction with the highest ATPase activity (corresponded to fraction 12 in dry embryos and fraction 20 in imbibed embryos).

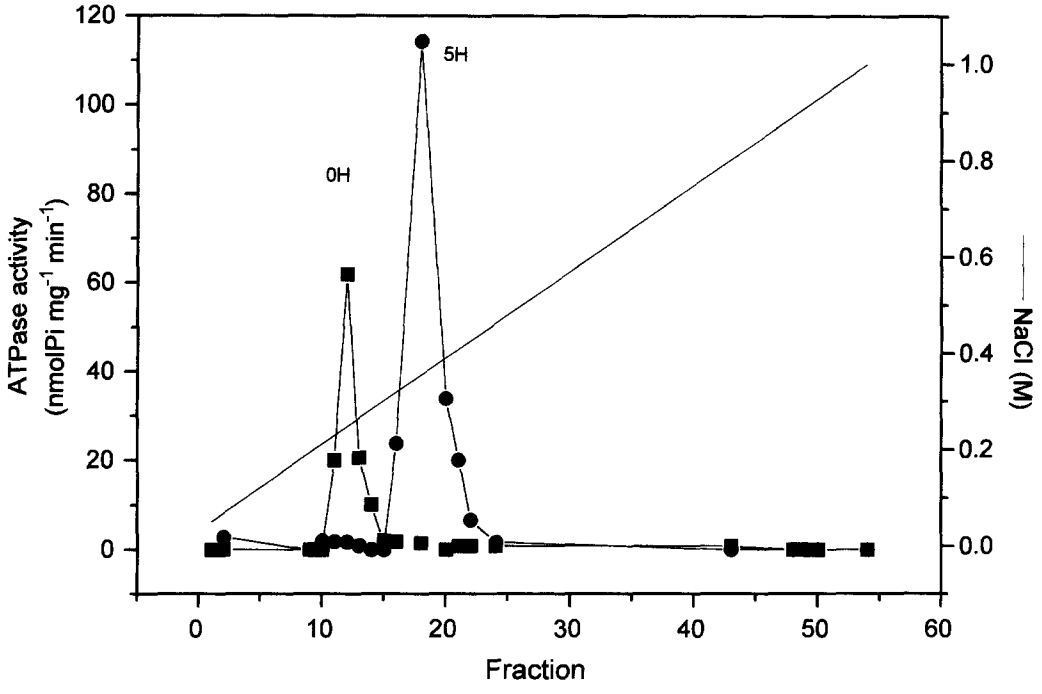

Fig. 6. Elution profile of the ATPase activity from a MonoQ 5/5 column from *n*-dodecyl-β-D-maltoside solubilized enzyme from 0 (■) and 5 (●) h imbibed embryos. The bound enzyme was eluted with a NaCl linear gradient in a buffer containing 10 mM histidine-HCl pH 6.5, 0.5 mM ATP, 0.1 mM DTT and 0.1 mM EDTA with a flow rate of 0.3 ml/min and 0.5 ml fractions were collected. An aliquot of 300 μl was supplemented with asolectin phospholipids to restore activity [23]. From this mixture, samples of 2 μg of protein were withdrawn to measure ATPase activity as described in Section 2.

relating the biochemical properties of the enzyme to different isoforms has been done by Palmgren and Christensen [42]. Their work established that the three AHA isoforms have different pH profile, vanadate sensitivity and LPC activation. In addition, an induced point mutation in an essential residue of the carboxy terminal segment of the yeast enzyme has been reported to shift the pH activity profile above 6.5 to a higher value [43]. Thus, the differences regarding values of optimal pH and p*K* shown by the enzymes of 0 and 5 h, may be due to differences in the isozyme expressed.

In relation to enzyme modification, it is well known that an increase in activity is promoted by phosphorylation of the enzyme [44,45]. Indeed, Eraso and Portillo, [46] showed that substitution of a negatively charged amino acid in the potential phosphorylation site of the yeast ATPase caused an increase in the activity and in the sensitivity to vanadate, and a displacement of the optimal pH to neutral values. On the other side, vanadate, an inhibitor that prevents the formation of the H^+-ATPase phosphoenzyme intermediate [47] is highly specific (K_i in the range of 1–10 μM). Mutations in and around the essential phosphorylation site in yeast H^+-ATPase caused an increase in the K_i for vanadate [43]. Our results show that the I_{50} values found for vanadate of the enzyme of 5 h and the enzyme from earlier times are markedly different. In addition, the difference found in thermal stability of the activities from 0 and 5 h may also be explained by distinct structural characteristics in both enzymes. This last difference and those observed in the pH profile and vanadate sensitivity of the two enzyme preparations would suggest possible changes in the amino acid sequence, or covalent modifications such as phosphorylation.

The differences in the properties of the enzyme at the times of imbibition tested could also reflect variations in the membrane environment, since it is well known that the activity of this enzyme is strongly influenced by the lipid surroundings [48]. In our sys-

tem, it is possible to expect changes in the lipid composition of the membrane during the early stages of germination, due to the presence of endogenous enzymes, i.e. lipoxygenases (EC 1.13.11.12) and fatty acid lyases [49].

Our data with LPC also show a difference in the pattern of activation of the enzyme, both in terms of maximal activation, and in terms of dependence of LPC concentration. Palmgren and Sommarin [32] and Palmgren et al. [50] reported that the range for both maximal activation and a complete detergent action by LPC was from 0.9 to 0.14 protein/LPC ratios. In our experimental conditions, the range of protein/LPC ratios explored was from 0.73 (at 30 μg LPC per ml) to 0.18 (at 120 μg LPC per ml), therefore, we considered that all the enzyme was fully activated by the LPC concentrations tested and had complete availability of substrate due to absence of latency release by Brij 58 addition. Olsson et al. [33] suggested that there is a direct interaction of LPC with the C-terminal of the ATPase, and Gomés et al. [51] proposed that LPC interacts with the membrane hydrophobic boundaries of the enzyme. In the same token, the smaller effect of trypsin on the 5 h enzyme could suggest that this enzyme was partially devoid of the C-terminal; however, according to the primary structure of the two maize isoforms reported so far [52,53], a removal of the C-terminal by trypsin cleavage would render proteins with a p*I* of 5.57 (Mha1) or 6.58 (Mha2). In both cases, the protein would not bind to the anion exchanger at pH 6.5 in our chromatographic conditions [54]. In fact, the 5 h enzyme eluted at a longer retention time than 0 h enzyme. Thus, the differences between the trypsin activation at the two times of imbibition could be interpreted as either structural differences in the protein or in the lipid composition of the membrane.

Our results with the MonoQ column showed closed, but defined, and different retention time peaks for the 0 and 5 h imbibition time samples. We have interpreted these data as an existence of different forms of the H^+-ATPase from plasma membrane, but the possibility that the differences found correspond to two types of ATPase was considered. Against this speculation, we observed that both the 0 and 5 h preparations reacted with specific anti-plasma membrane H^+-ATPase antibodies giving the same band of about 100 kDa (results not shown). The other possible ATPase present was the plasma membrane Ca^{2+}-ATPase, since endomembranes are in very low amounts in these preparations [20]. This Ca^{2+} pump has a characteristic calcium-dependent ITPase activity, which in our 0 and 5 h preparations was similar (0.7 and 1.1 nmol ITP hydrolyzed/mg/min, respectively), and very low as compared to the analogous activity from radish roots (16.7 nmol ITP hydrolyzed/mg/min, [28]). This result argues against the possibility that the Ca^{2+}-ATPase was one of the two enzymes.

The nature of the dissimilarity (covalent modification, isoforms) was not studied in this work; nonetheless, the difference in the elution profiles of the solubilized enzyme obtained from embryos imbibed 0 and 5 h, is consistent with the different responses of the activity to pH, temperature, vanadate, LPC and trypsin.

So far, there are reports documenting the differential expression of the H^+-ATPase isoforms at the level of mRNA in different tissues [36,55–57] or the characterization of some isoforms expressed in yeast endoplasmic reticulum [42]. In our work, we describe two distinct ATPase activities based on a biochemical characterization in the native membranes from imbibed and non-imbibed embryos. These differences could be explained in terms of two distinct forms of the enzyme or due to different membrane environments of the protein in the onset of germination. Such behavior of the enzyme is relevant in order to know the dynamics of the membrane components in the transition from the dry to the hydrated state of the seed embryo.

Acknowledgements

The authors are indebted to Drs. Armando Gómez-Puyou and Diego González-Halphen for the review of the manuscript and Irma Bernal-Lugo for helpful discussions on this work. They thank Consuelo Enríquez-Arredondo for excellent technical assistance. The technical support of Laurel Fabila Ibarra is also recognized. This work was supported by Grants 4836-N9406 from CONACyT and IN204097 from DGAPA, UNAM, Mexico. S.S.N. has a fellowship by DGAPA, UNAM.

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