Inhibition of Maize Root H^+-ATPase by Fluoride and Fluoroaluminate Complexes[1]

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Vesicles derived from maize roots retain a membrane-bound H^+-ATPase that is able to pump H^+ at the expense of ATP hydrolysis. The H^+ pumping and the ATPase activity of these vesicles are inhibited by lithium fluoride and by the complex formed between fluoride and aluminum. The inhibition promoted by lithium fluoride increases as the $MgCl_2$ concentration in the medium is increased from 2 to 20 mM. The inhibitory activity of both lithium fluoride and aluminum fluoride increases as the temperature of the medium is increased from 20 to 35°C. Inorganic phosphate (10–40 mM) inhibits the H^+-ATPase at pH 6.5 but not at pH 7.0, and at both pH values, it antagonizes the inhibition promoted by lithium fluoride and fluoroaluminate complexes.

The presence of aluminum in acid soils inhibits plant growth (Kinraide, 1991). Although the mechanism of aluminum toxicity is not yet understood, Kasai et al. (1992) found that aluminum stress promotes an inhibition of the plasma membrane H^+-ATPase of barley roots. Tu and Brouillette (1987) observed that aluminum inhibits the plasma membrane H^+-ATPase of corn root, and this enzyme is a P-type transport ATPase that plays a key role in nutrition and development of plants (Slayman, 1974; Perlin et al., 1984; Briskin and Hanson, 1992). In the soil, aluminum forms complexes with different anions, including fluoride, OH^-, phosphate, and silicate (Kinraide, 1991). Fluoride anions and fluoroaluminate complex inhibit two different animal P-type ATPases, namely the sarcoplasmic reticulum Ca^{2+}-ATPase and the plasma membrane Na^+/K^+ ATPase (Robinson et al., 1986; Missiaen et al., 1989; Murphy and Coll, 1992; Murphy and Hoover, 1992; Troullier et al., 1992). The inhibitory activity of fluoride varies, depending on the Mg^{2+} concentration in the medium. Murphy and Coll (1992) and Murphy and Hoover (1992) proposed that this inhibition is promoted by the binding of free fluoride to the magnesium bound to the enzyme. The fluoroaluminate complex seems to act as a Pi analog that strongly binds to the E_2 conformation of the two transport ATPases in a quasi-irreversible process that

cannot be overcome by high Pi concentrations (Robinson et al., 1986; Missiaen et al., 1989; Troullier et al., 1992). Recently, it was shown that fluoroaluminates inhibit the H^+-ATPase purified from yeast plasma membrane (Rapin-Legroux et al., 1994). In plants, Magyar et al. (1989) observed that fluoroaluminate inhibits the vanadate-sensitive ATPase activity found in wheat root microsomes. In this work we studied the inhibition of the plasma membrane H^+-ATPase from corn (*Zea mays* L.) roots by fluoride and fluoroaluminate complexes.

MATERIALS AND METHODS

Plant Material

Corn (*Zea mays* L.) seeds were germinated in the dark at 28°C on wet filter paper. The roots were harvested after the 4th d of growth.

Isolation of Plasma Membrane Vesicles

Plasma membrane vesicles were isolated from corn roots by differential centrifugation and Suc gradient as previously described (Nagao et al., 1987; Serrano, 1988). Briefly, 100 g of roots were homogenized in a blender with 150 mL of an ice-cold buffer containing 0.6 M Suc, 1.3 M KCl, 17 mM EDTA, 3.3 mM DTT, 0.13% (w/v) BSA, 0.7 mM PMSF, and 167 mM Tris-HCl buffer (pH 8.0). The homogenate was strained through two layers of cheesecloth and centrifuged at 8,000g for 10 min. The supernatant was centrifuged at 100,000g for 30 min. The pellet was resuspended in a small volume of ice-cold buffer containing 10 mM Tris-HCl (pH 7.6), 20% (v/v) glycerol, 1 mM DTT, and 1 mM EDTA. The suspension was layered over a 32.5 and 46.5% (w/v) discontinuous Suc gradient and centrifuged at 35,000 rpm for 5 h in a Beckman SW 40 rotor. The gradient solutions, in addition to Suc, contained 10 mM Tris-HCl buffer (pH 7.6), 1 mM DTT, and 1 mM EDTA. The vesicles that sedimented at the interface between 32.5 and 46.5% Suc were collected, diluted with 3 volumes of ice-cold water, and centrifuged at 100,000g for 30 min. The pellet was resuspended in a medium containing 10 mM Tris-HCl (pH 7.6), 20% (v/v) glycerol, 1 mM DTT, and 1 mM EDTA. The vesicles were then frozen under liquid nitrogen and stored at −70°C until use.

Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; FCCP, carbonyl cyanide *p*(trifluoromethoxy)-phenylhydrazone.

Protein concentrations were determined by the method of Lowry et al. (1951), with BSA as the standard.
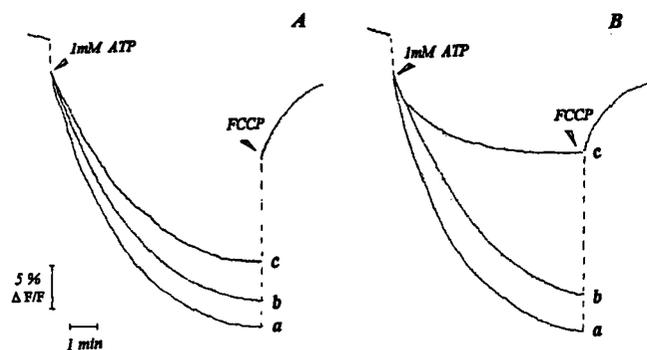
ATPase Activity

To avoid fluoride contamination derived from glass, all assays were performed using plastic test tubes. ATPase activity was determined by measuring the release of Pi, either colorimetrically (Fiske and Subarow, 1925) or using $[\gamma\text{-}^{32}P]$ATP as previously described (de Meis, 1988). A large fraction (85–95%) of the vesicle ATPase activity measured at pH 6.5 was inhibited by vanadate, an inhibitor of the H^+-ATPase (Palmgren, 1990). In all experiments, the ATPase activity was measured with and without 0.1 mM vanadate and the difference between these two activities was attributed to the H^+-ATPase. This activity was not impaired by 50 mM KNO_3, an inhibitor of tonoplast ATPase, and 1 mM molybdate, an inhibitor of phosphatases (Palmgren, 1990).
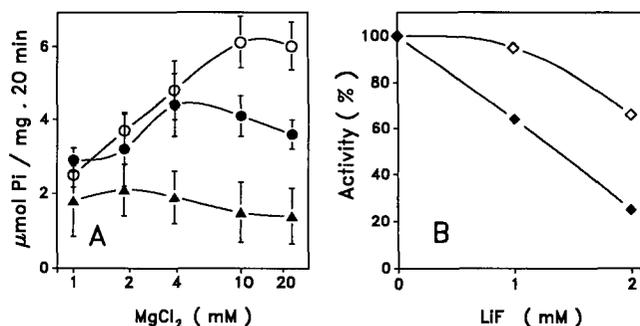
Proton Uptake

The accumulation of protons inside the vesicles was determined by measuring the fluorescence quenching of ACMA with an Hitachi (Tokyo, Japan) F-3010 fluorometer. The excitation wavelength was set at 415 nm and the emission wavelength was set at 485 nm. In different vesicle preparations tested, the inclusion of 0.1 mM vanadate in the assay medium promoted a 85 to 95% inhibition of the fluorescence quenching measured after the addition of ATP (Ramos et al., 1994).

Materials

ATP disodium salt and FCCP were purchased from Sigma. All other reagents used were of analytical grade. A
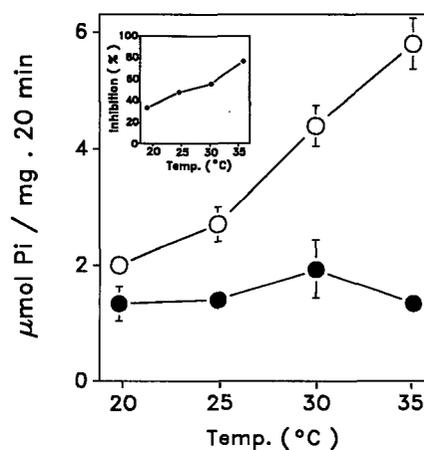

Figure 2. Inhibition by fluoride: magnesium dependence. The assay medium composition was 50 mM Mops-Tris buffer (pH 6.5), 1 mM ATP, and the magnesium chloride concentrations shown in the figure. The reaction was started by the addition of 30 μg/mL plasma membrane protein and was arrested after 20 min of incubation at 35°C by the addition of TCA (10%, w/v). A, control (O); 1 mM lithium fluoride (●); 2 mM lithium fluoride (▲). B, The inhibition promoted by fluoride in presence of 2 mM (◇) and 10 mM (◆) magnesium chloride was calculated using the experimental values from A. Values are means ± SE of five experiments.

500 mM Pi-Tris stock solution adjusted at different pH values was prepared by mixing aqueous solutions of phosphoric acid and Tris. A 10 mM stock solution of FCCP in ethanol was used in all experiments. The final concentration of ethanol in the assay medium never exceeded 0.03%.

RESULTS

Inhibition by Fluoride and Magnesium

This was observed using high magnesium chloride concentrations (4–10 mM) and 2 mM lithium fluoride. In these

Figure 1. Inhibition of H^+ pumping by fluoride (A) and aluminum fluoride (B). The composition of the control medium (a) was 10 mM Mops-Tris buffer (pH 6.5), 4 mM magnesium chloride, 2 μM ACMA, and 0.2 mg/mL plasma membrane protein. The reaction was performed at room temperature (25°C), and it was started by the addition of ATP to a final concentration of 1 mM. At the end of the experiment 5 μM FCCP, a proton ionophore, was added. A, The vesicles were preincubated with 1 (b) or 2 (c) mM lithium fluoride for 10 min prior to the addition of ATP. B, The vesicles were preincubated for 10 min with either 0.1 mM aluminum chloride (b) or 0.1 mM aluminum chloride plus 1 mM lithium fluoride (c). The figure shows a representative experiment. Essentially the same results were obtained in three experiments using different vesicles preparations.

Figure 3. Effect of temperature on the inhibition promoted by fluoride. The assay medium composition was 50 mM Mops-Tris buffer (pH 6.5), 1 mM ATP, and 10 mM magnesium chloride. The reaction was started by the addition of 30 μg/mL plasma membrane vesicles and was arrested after 20 min by the addition of TCA. The reaction was carried at different temperatures either in the absence (O) or presence (●) of 2 mM lithium fluoride. Inset, The percentage of inhibition by magnesium fluoride. Values are means ± SE of three experiments.
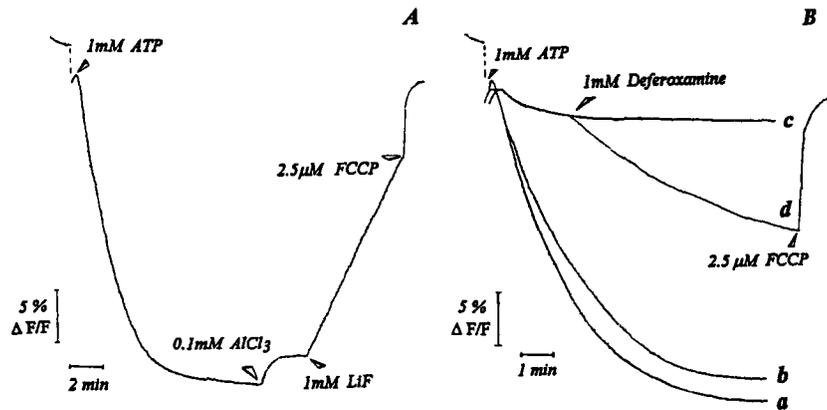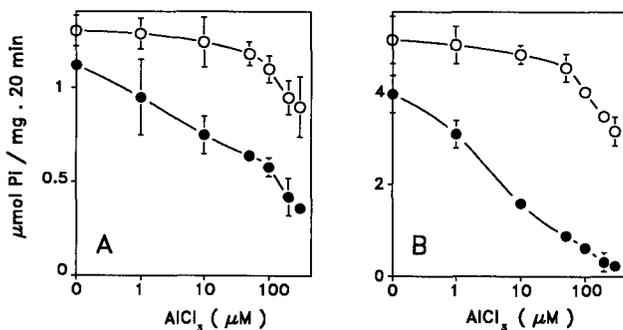
Figure 4. Inhibition of H⁺ pumping by fluoroaluminate complexes: effect of deferoxamine. The assay medium composition was 10 mM Mops-Tris buffer (pH 6.5), 100 mM KCl, 2 mM magnesium chloride, 2 μM ACMA, and 0.2 mg/mL plasma membrane protein. The reaction was performed at room temperature (25°C), and it was started by the addition of ATP to a final concentration of 1 mM. A, 0.1 mM aluminum chloride, 1 mM lithium fluoride, and 2.5 μM FCCP were added as indicated. B, Control without additions (a), 0.2 mM aluminum chloride, 2 mM lithium fluoride, and 1 mM deferoxamine were added to the assay medium (b). The vesicles were preincubated in this medium for 10 min before the addition of ATP. c, Same as b but without deferoxamine. d, 0.2 mM aluminum chloride and 2 mM lithium fluoride were added to the assay medium. The vesicles were preincubated in this medium for 10 min before the addition of ATP. Deferoxamine (1 mM) and FCCP (2.5 μM) were added as indicated. Essentially the same results were obtained in two experiments using different vesicle preparations.

conditions, both the H⁺ pumping (Fig. 1A) and the ATPase activity (Fig. 2) of the vesicles were inhibited. The degree of inhibition varied, depending on the magnesium and the fluoride concentrations used (Fig. 2). Both the activity of the H⁺-ATPase and the inhibitory activity of fluoride increased as the temperature of the medium was increased from 20 to 35°C (Fig. 3).
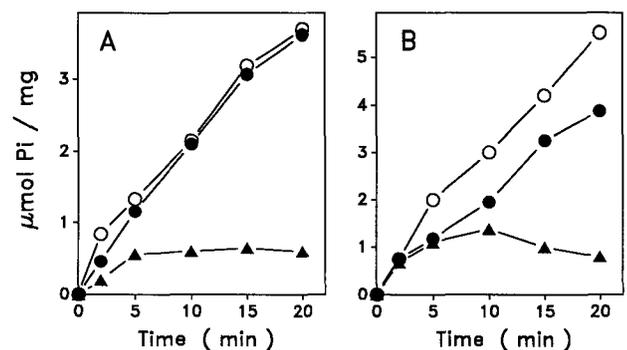
Inhibition by Aluminium and Fluoroaluminate Complex

In these experiments, a low concentration of magnesium chloride (2 mM) was used to minimize the effect of fluoride anions. In agreement with Tu and Brouillette (1987), an inhibition of the pumping activity of the H⁺-ATPase was observed when aluminum chloride was added to the me-

dium, and this inhibition was greatly enhanced in the presence of fluoride (Figs. 1B, 4, and 5) and was observed both with and without 100 mM KCl (cf. Figs. 1 and 4). During proton accumulation, both an electrogenic ($\Delta\psi$) and pH (ΔpH) gradient were formed across the vesicles membrane. The $\Delta\psi$ was practically abolished in the presence of 100 mM KCl (Perlin et al., 1984). Deferoxamine, a chelator of aluminum, reversed the inhibition promoted by aluminum fluoride (Figs. 4B and 6). Notice in Figure 6B that deferoxamine did not abolish completely the inhibition measured in the presence of a high concentration of magnesium. This residual inhibition was related to the com-

Figure 5. Inhibition of the H⁺-ATPase activity by fluoroaluminate complex at 20 and 35°C. The assay medium composition was 50 mM Mops-Tris buffer (pH 6.5), 1 mM ATP, 2 mM magnesium chloride, and 3 μM/mL plasma membrane protein. The reaction was performed at 20 (A) and 35°C. (B). Control without additions (O) and with 1 mM lithium fluoride (●). Values are means ± SE of five experiments.

Figure 6. Inhibition of the H⁺-ATPase activity by fluoroaluminate complex: effect of deferoxamine. The assay medium composition was 50 mM Mops-Tris buffer (pH 6.5), 1 mM ATP, 30 μg/mL plasma membrane protein, and either 2 mM magnesium chloride (A) or 10 mM magnesium chloride (B). 0.5 mM deferoxamine (O); 0.1 mM aluminum chloride, 1 mM lithium fluoride, and 0.5 mM deferoxamine (●); 0.1 mM aluminum chloride and 1 mM lithium fluoride (▲). Essentially the same results were obtained in two experiments using different vesicle preparations.
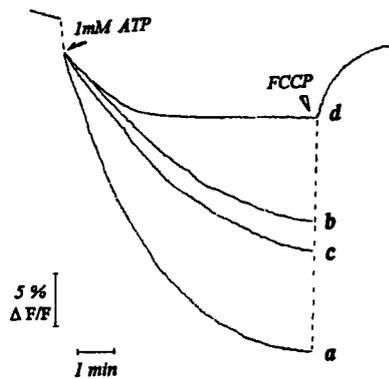
Figure 7. Effect of Pi on H^+ pumping. The assay medium composition was 10 mM Mops-Tris buffer (pH 6.5), 4 mM magnesium chloride, 2 μM ACMA, and 0.2 mg/mL plasma membrane protein. The reaction was performed at room temperature (25°C) and was started by the addition of ATP to a final concentration of 1 mM. Prior to the addition of ATP, the vesicles were preincubated for 10 min with 0.1 mM aluminum chloride and 1 mM lithium fluoride, both in the absence and in the presence of 40 mM Pi. a, Control without addition; b, with Pi; c, the vesicles were preincubated with aluminum chloride, lithium fluoride, and Pi; d, vesicles were preincubated with aluminum chloride and lithium fluoride. Essentially the same results were obtained in two experiments using different vesicle preparations.

bined effects of fluoride and magnesium shown in Figure 2 and not to an effect of small concentrations of fluoroaluminate complex that might still be formed in the presence of deferoxamine (cf. A and B in Fig. 6). The inhibition promoted by the fluoroaluminate complex increased as the temperature of the medium was increased from 20 to 35°C. In the presence of 1 mM lithium fluoride, half-maximal inhibition was observed with the addition of 100 μM aluminum chloride at 20°C (Fig. 5A) and in presence of 8 μM aluminum chloride at 35°C (Fig. 5B). The small inhibition promoted by aluminum in the absence of fluoride did not vary with the temperature. In five different experiments, the inhibition promoted by 0.3 mM aluminum chloride at

Figure 8. Effect of Pi at different pH values. The assay medium composition was 50 mM Mops-Tris buffer adjusted to either pH 6.5 (a) or pH 7.0 (b), 1 mM ATP, 2 mM magnesium chloride, 30 μg/mL plasma membrane protein, and the Pi concentrations indicated. Control (O), 2 mM lithium fluoride (▲), and 0.1 mM aluminum chloride plus 1 mM lithium fluoride (●). Values are means ± SE of three experiments.

20 and 35°C were of 41.1 ± 7.3 and 45.4 ± 4.8%, respectively, (mean ± SE).
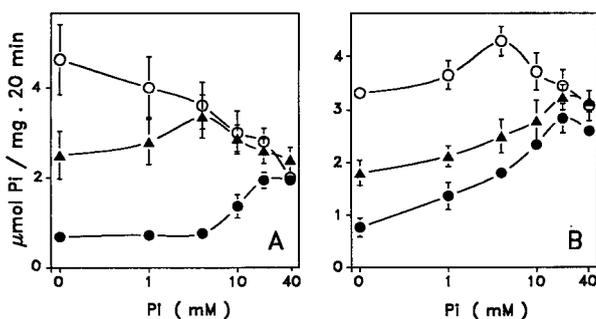
Effect of Phosphate and pH

High Pi concentrations inhibited both the H^+ uptake (Fig. 7) and the hydrolysis of ATP (Fig. 8A) by the maize H^+-ATPase. This inhibition was observed at both pH 6.0 (data not shown) and pH 6.5 (Fig. 8A) but was no longer detected when the pH of the medium was increased to 7.0 (cf. A and B in Fig. 8). The inhibition of the H^+-ATPase promoted by fluoride and by the fluoroaluminate complex was antagonized by Pi at all pH values tested (Figs. 7 and 8). In a control experiment it was found that the inhibitory activities of fluoride and aluminum fluoride did not vary when the concentration of Mops-Tris buffer (pH 6.5) was increased to 100 mM (data not shown).

DISCUSSION

Fluoride Inhibition

The different P-type transport ATPases can be found in two different conformations named E_1 and E_2. During the catalytic cycle the form E_1 is phosphorylated by ATP. The enzyme form E_2 binds Mg^{2+} and the complex $E_2 \cdot Mg$ is phosphorylated by Pi (de Meis and Vianna, 1979; Pedersen and Carafoli, 1987; de Meis, 1989; Vara and Medina, 1990). Similar to the Ca^{2+}-ATPase (Coll and Murphy, 1992; Murphy and Coll, 1992) the inhibition of the H^+-ATPase of corn roots by fluoride is enhanced by Mg^{2+} (Fig. 2) and is antagonized by Pi (Fig. 8). This suggests that fluoride interacts with the enzyme to form E_2 of the H^+-ATPase. Kinetics evidence indicates that for the Ca^{2+}-ATPase the inhibition is promoted by the binding of fluoride to the complex $Mg \cdot E_2$ (Coll and Murphy, 1992; Murphy and Coll, 1992). Our findings indicate that the magnesium fluoride complex inhibits the H^+-ATPase but the data available at present are not sufficient to exclude the possibility that the binding of F^- to the enzyme $Mg \cdot E_2$ is also involved in the inhibition of the enzyme. In the presence of 2 mM lithium fluoride, the concentration of MgF^+ available after the

Table I. *Association constants for different ionic reactions (Hogfeldt, 1982; Alva and Sumner, 1989; Sikora et al., 1992)*

Reaction	log K_a
$Al^{3+} + 2 F^- \rightleftharpoons AlF_2^+$	12.7
$Al^{3+} + 3 F^- \rightleftharpoons AlF_3$	16.8
$Al^{3+} + 4 F^- \rightleftharpoons AlF_4^-$	19.4
$Al^{3+} + 5 F^- \rightleftharpoons AlF_5^{2-}$	20.6
$Al^{3+} + HPO_4^{2-} \rightleftharpoons AlHPO_4^+$	8.1[a]
$Al^{3+} + H_2PO_4^- \rightleftharpoons AlH_2PO_4^{2+}$	4.5[a]
$Mg^{2+} + F^- \rightleftharpoons MgF^+$	1.8
$Mg^{2+} + HPO_4^{2-} \rightleftharpoons MgHPO_4$	1.8

[a] Values were calculated as previously described (de Meis et al., 1985) using the equilibrium constants reported by Sikora et al. (1992) and Alva and Sumner (1989) for the reactions that include proton dissociation from the phosphate anion: $Al^{3+} + PO_4^{3-} + H^+ \rightleftharpoons AlHPO_4^+$ (log K_a 19.3 and 19.8) and $Al^{3+} + PO_4^{3-} + 2H^+ \rightleftharpoons AlH_2PO_4^{2+}$ (log K_a 22.55 and 22.7).

addition of 10 mM magnesium chloride is 0.7 mM. This was calculated using the stability constant for MgF$^+$ shown in Table I.

Inhibition by Aluminum Fluoride

Tu and Brouillette (1987) estimated that at pH 6.6 the dissociation constant of the Al^{3+}-ATP complex is 0.83 mM and that of Mg^{2+}-ATP is 0.05 mM. Thus, as proposed by these authors, it is unlikely that the Al^{3+}-ATP complex functions as a competitive inhibitor to the Mg^{2+}-ATPase activity under the conditions used in this work. The phosphorylation of the Ca^{2+}-ATPase by Pi is inhibited by fluoroaluminate. This observation led to the proposal that the fluoride complex interacts with the E$_2$ form (Missiaen et al., 1989; Troullier et al., 1992). We were not able to measure phosphorylation of the H$^+$-ATPase by Pi, but the finding that Pi antagonizes the inhibition promoted by aluminum fluoride suggests that, as in the other P-type ATPases, the fluoroaluminate complex interacts with the Pi-binding site of the H$^+$-ATPase E$_2$ form. The effect of Pi observed in Figures 7 and 8 could also be related to the formation of aluminum phosphate complexes that would lead to a decrease of fluoroaluminate concentration in the assay medium. This possibility is excluded by the large difference of the K_a for the formation of the complexes of aluminum with phosphate and with fluoride (Table I). Aluminum can form different complexes with fluoride anions (Antonny and Chabre, 1992). At present, it is not yet clear which complex inhibits the different P-type ATPases. Plots of activity as a function of the calculated concentrations of the different complexes that aluminum can form with fluoride are not conclusive (Troullier et al., 1992; Rapin-Legroux et al., 1994). Troullier et al. (1992) measured the amount of fluoride and aluminum that remains attached to the Ca^{2+}-ATPase after thermal denaturation and found 4.8 nmol of Al^{3+} and 19.6 nmol F$^-$/mg protein. These values led the authors to propose that AlF$_4^-$ is the inhibitory complex of the Ca^{2+}-ATPase.

Effect of Temperature

The equilibrium between the forms E$_1$ and E$_2$ of different P-type ATPases varies with the temperature. The fraction of enzyme found in the E$_2$ form is larger as the temperature is increased from 20 to 35°C (Masuda and de Meis, 1977; Pick and Karlish, 1982). The effect of the temperature observed in Figures 3 and 5 may indicate that, similar to the other transport ATPases, the ratio between the enzyme forms E$_2$/E$_1$ of the H$^+$-ATPase also increases as the temperature is increased from 20 to 35°C, thus favoring the interaction of fluoride and fluoroaluminate complex with the enzyme form E$_2$. As far as we know, the effects of temperature and protection by Pi have not been observed in earlier works with either plant microsomes or yeast H$^+$-ATPase.

Effect of Pi

In a previous report (Goffeau and de Meis, 1990) it was shown that the H$^+$-ATPase purified from plasma membrane of *Schizosaccharomyces pombe* is activated by Pi (10–80 mM). The degree of activation increased when the pH of the assay medium was increased from 6.0 to 7.0 and was abolished after substitution of a single amino acid of the enzyme β-strand (Lys^{250} → Thr). In contrast with this finding, in this report it was found that Pi inhibits the H$^+$-ATPase of maize roots at pH 6.5 and has practically no effect at pH 7.0 (Fig. 6). It may be possible that the different effects of Pi obtained with the H$^+$-ATPase from yeast and from maize roots are related to a small difference in the amino acid sequence between the two enzymes.

Finally, the strong inhibition of the H$^+$-ATPase observed in medium containing fluoride and aluminum may suggest that fluoroaluminate could play a role in the mechanism of aluminum toxicity and inhibition of plant growth observed in acid soils. However, at present we do not know whether fluoroaluminate is able to penetrate the cells of the corn roots (Kinraide, 1991).

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