Plant Physiol. (1991) 96, 1345–1353
0032-0889/91/96/1345/09/$01.00/0

Received for publication January 15, 1991
Accepted April 30, 1991

Differentiation between Several Types of Phosphohydrolases in Light Microsomes of Corn Roots[1]

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ABSTRACT

The phosphohydrolase activity of a light microsomal fraction isolated from corn roots (*Zea mays* L. cv LG 55) was investigated. The fraction, which appears to be enriched in endoplasmic reticulum and Golgi membranes, has ATPase and pyrophosphatase activities that hydrolyze ATP and pyrophosphate at an optimum pH of 7.0, with K_m values of about 160 and 240 micromolar and with V_{max} values of about 200 and 50 nanomoles substrate hydrolyzed per milligram protein per minute, respectively. These enzymes differ in their sensitivity to anions and inhibitors. The ATPase is stimulated by sulfate anions, whereas pyrophosphatase is inhibited by molybdate. Furthermore, the simultaneous addition of ATP and pyrophosphate to the reaction medium increases phosphohydrolysis, suggesting that separate enzymes are operating in the membranes. We also observed that pyrophosphate competitively inhibits the ATPase, whereas ATP has no significant effect on the pyrophosphatase. On the other hand, we observed a detergent-stimulated, molybdate-insensitive inosine diphosphatase activity which, in the native state, hydrolyzes inosine diphosphate with a K_m of about 700 micromolar and a V_{max} of about 450 nanomoles inosine diphosphate hydrolyzed per milligram protein per minute. In the solubilized form, the enzyme appears to be fully active, exhibiting lower K_m values to hydrolyze inosine diphosphate. Furthermore, we found that native inosine diphosphatase is inhibited either by ATP or pyrophosphate, whereas inosine diphosphate inhibits the ATPase, but has no significant effect on the pyrophosphatase. It appears that inosine diphosphate is a positive modulator of the inosine diphosphatase, whereas ATP and pyrophosphate act as negative modulators of this enzyme.

The primary event for transport of solutes across the plasma and vacuolar membranes of plant cells is considered to be the active translocation of H^+ (23). Proton translocation generates an electrochemical potential that functions as the driving force for the transport of anions, cations, amino acids, and sugars. It has been demonstrated that ATP energizes the H^+ transport across membranes of plant cells (27), but the involvement of other phosphoesters, such as PPi and ADP, has been implicated in proton motive force generation (4, 18, 25, 32).

Corn root (*Zea mays* L. cv LG55) microsomal fractions contain a K^+-stimulated, vanadate-inhibited H^+-ATPase, which is localized in plasma membranes (2, 7, 9), and a Cl^--stimulated, nitrate-inhibited H^+-ATPase, which is a tonoplast enzyme (8, 22). Furthermore, another proton pumping ATPase has been identified on the Golgi membranes of corn coleoptiles (3), and, recently, we observed a sulfate-stimulated ATPase in light microsomes of corn roots (29).

Pyrophosphate-driven proton pumps have been found in a tonoplast-enriched fraction and in a Golgi-enriched fraction of maize coleoptiles (4), as well as in the tonoplast of red beet storage roots (25, 26), oat roots (32), and corn roots (5). However, coupling between this proton transport and PPase[2] is not well defined. Furthermore, it is not well established whether in the various subcellular fractions, PPase and ATPase activities derive from the same enzyme or from distinct enzymes. The different detergent solubilities of the PPase and ATPase (30), as well as their different ion requirements (13, 31), sensitivity to inhibitors (32), and additive activities (4), indicate that membrane-associated PPase and ATPase are distinct phosphohydrolases. Furthermore, in tonoplast vesicles of oat roots and red beet roots, they were physically separated by chromatography of detergent-solubilized membranes in Sepharose CL-GB columns (32) and Sephacryl-400 columns (26), respectively.

On the other hand, IDPase has been reported as an exclusive marker for Golgi membranes of higher plant cells (10, 19, 20, 24). However, several NDPases have been found also in other cell constituents such as the endoplasmic reticulum, plasma membrane, and tonoplast (10). Although the function of these enzymes is not well known, it has been suggested that NDPases are involved in the synthesis or secretion of polysaccharide-rich products (10).

In this work, we studied the phosphohydrolase activity in a light microsomal fraction of corn roots, and investigated the individuality of the enzymes that utilize different energy-rich substrates such as ATP, IDP, and PPi. The results indicate that ATP, IDP, and PPi are hydrolyzed by distinct enzymes (ATPase, IDPase, and PPase), and that PPase is not significantly altered by the presence of nucleotides, whereas the ATPase and IDPase appear to be regulated by the cellular energy levels expressed by the PPi, IDP, and ATP content.

MATERIALS AND METHODS

Plant Material

Seeds of maize (*Zea mays* L. cv LG 55) were surface-sterilized in a 7% (w/v) solution of commercial bleach, rinsed

[1] Supported by grants from the National Institute for Scientific Research of the Portuguese Ministry of Education and from the Calouste Gulbenkian Foundation.

[2] Abbreviations: PPase, pyrophosphatase; IDPase, inosine diphosphatase; NDPase, nucleoside diphosphatase.

with tap water, and soaked in running tap water for about 24 h. Seeds were then germinated on damp paper towels in plastic trays in a darkened growth cabinet for 4 to 5 d at 25°C.

Preparation of Microsomal Vesicles

Entire primary roots were excised from seedlings and chopped into 1 to 0.5 cm segments in the isolation medium containing 250 mM sucrose, 1 mM EDTA, 1 mM DTT, 100 μM PMSF, 25 mM Tris, and 25 mM Mops (pH 7.6). The roots were ground with a chilled mortar and pestle and then homogenized in a Waring Blendor for 20 s in the isolation medium (2 mL/g fresh weight). After filtration through two layers of cheesecloth, the homogenate was centrifuged at 10,000*g* for 20 min. The mitochondrial pellet was discarded, and the supernatant was centrifuged again at 80,000*g* for 1 h. The microsomal pellet was suspended in a buffer solution containing 250 mM sucrose, 0.1 mM EDTA, 5 mM Tris, and 5 mM Mops (pH 7.0).

Isolation of Light Microsomes

After diluting the microsomal preparation to about 24 mL with the suspension buffer, aliquots of 4 mL were layered on the top of 30 mL discontinuous sucrose density gradients (16:34:40%, w/w) containing 5 mM Tris and 5 mM Mops (pH 7.0). Then, the material was centrifuged at 90,000*g* for 3 h. The light membrane fraction, collected onto the 16% sucrose layer, was diluted with 5 mM Tris and 5 mM Mops buffer (pH 7.0), and it was centrifuged at 80,000*g* for 1 h. Finally, the sediments were resuspended in 250 mM sucrose, 5 mM Tris, 5 mM Mops (pH 7.0), and 1 mM DTT to a final concentration of 3 to 8 mg protein/mL. All steps were carried out at 2 to 5°C. The protein was measured by the biuret method (16), using BSA as standard.

The isolated microsomes were frozen in liquid air to be stored at −30°C. Under these conditions, no significant loss of activity was observed for several weeks. The activity was essentially decreased when the initial steps of the isolation procedure were delayed.

The heavier microsomal fractions obtained at the 16/34% and 34/40% interfaces of the sucrose gradients (F34 and F40) were also collected for characterization of the membranes and analysis of their distribution in the gradient.

Measurement of Phosphohydrolase Activities

ATPase, IDPase, and PPase activities were determined by measuring the Pi liberation associated with hydrolysis of ATP, IDP, and PPi, respectively. Membrane vesicles (0.15–0.3 mg protein) were incubated in 2 mL of 3 mM $MgSO_4$, 25 mM K_2SO_4, 25 mM Tris, and 5 μg/mL oligomycin at pH 7.0. The reaction was started by adding various concentrations of substrate (ATP, IDP, or PPi), and it was allowed to proceed for 15 min at 30°C. It was stopped by adding 0.1 mL of ice-cold 10% (w/v) TCA. After 2 min on ice, the precipitated protein was removed by centrifugation, and the supernatants were assayed for Pi by the method of Taussky and Shorr (28). When Pi liberation was measured in the presence of Triton X-100, SDS was included in the color reagents to eliminate detergent interference (21). The PPase activity was calculated as half the rate of PPi liberation, because the hydrolysis of 1 mol of PPi yields 2 mol of Pi. For the acid phosphatase assay, *p*-nitrophenylphosphate was used as substrate. Controls without protein were performed to obtain correct values of enzyme activity.

In some experiments, the ATPase activity was isotopically determined according to the method of Griffith *et al.* (11). The reaction was started by adding 80 μg of membrane protein to 500 μL of incubation medium containing 50 mM KCl, 5 mM $MgSO_4$, 30 mM Tris/Mes (pH 7.0), and 2 mM Tris-[^{32}P]ATP (0.2 mCi/mmol) at 30°C. Fifteen minutes later, the reaction was stopped and the excess of [^{32}P]ATP was removed by adding 720 μL of 15% charcoal Norit A in 5% (w/v) TCA. The suspensions obtained were kept on ice for 30 min and then centrifuged for 3 min in an Eppendorf microfuge. Finally, aliquots of 400 μL supernatants were collected into 9 mL of scintillation cocktail for counting radioactivity in a Packard Tricarb liquid scintillation spectrophotometer, model 460-CD. The composition of the scintillation fluid per liter of toluene was 7.3 g 2,5-diphenyloxazole, 176 mg *p*-bis[2-(5-phenyloxazolyl)] benzene, and 250 mL Triton X-100.

Controls without protein were performed for correction of the ATPase measurements.

Analysis of Membrane Marker Enzymes

The vanadate-inhibited ATPase and the nitrate-inhibited ATPase were assayed as marker enzymes for the plasma membranes and the tonoplast membranes, respectively.

The reactions were performed as described above in the presence of 50 μM orthovanadate or 50 mM KNO_3 plus vanadate, respectively.

The endoplasmic reticulum was identified by measuring the activity of the NADPH-Cyt *c* reductase. The reaction was performed at 30°C by monitoring the reduction of Cyt *c* at 550 nm in a double beam Varian Techtron spectrophotometer, model 635, connected to a Perkin-Elmer 561 recorder (12). The reaction medium (3 mL) contained 150 μg of protein, 1.7 mM potassium cyanide, 3 μM antimycin A (if present), 30 μM Cyt *c*, and about 40 mM phosphate buffer (pH 7.5). The reaction started with the addition of 100 μM NADPH. Controls, either with boiled enzyme or without NADPH, were performed and no Cyt *c* reduction was obtained.

Treatment of the Data

All results represent the mean values obtained from three or four repetitive experiments.

Chemicals

All reagents were analytical grade. Pyrophosphate was obtained from British Drug House Chemicals Ltd; ATP and IDP were purchased from Sigma; [^{32}P]ATP from Amersham.

RESULTS

Characterization of ATPase and PPase Activities in Light Microsomes of Corn Roots

The light microsomal fraction isolated from corn roots was studied with respect to its phosphohydrolase activity. Figure

1 shows that light microsomes displayed an ATPase activity that was higher in a sulfate-containing medium than in a chloride-containing medium. Furthermore, it was not sensitive to Ca^{2+} over Mg^{2+}; it was stimulated by detergents and it was inhibited by the anion transport blocker, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). It appears that sulfate-stimulated ATPase is an intrinsic component of the membrane, which has high sensitivity to DIDS. As shown in Figure 2A, ATP hydrolysis followed the Michaelis-Menten kinetics in a sulfate-containing medium. The ATPase activity was increased as the Mg-ATP concentration increased in the medium up to about 1 mM. Hanes-Woolf analysis showed that the ATPase had an apparent K_m for Mg-ATP of about 160 μM and a V_{max} of about 180 nmol Pi mg protein^{-1} min^{-1} (Fig. 2A, inset).

The light microsomal fraction of corn roots also had PPase activity (Fig. 2B). The values obtained for K_m (PPi) were about 240 μM and those obtained for V_{max} were about 100 nmol Pi mg protein^{-1} min^{-1}, which correspond to 50 nmol PPi hydrolyzed mg protein^{-1} min^{-1}. Therefore, it appears that PPase has lower affinity and lower capacity to react with PPi as compared with the ability of ATPase to react with ATP.

To investigate whether these phosphohydrolase activities derive from the same enzyme or from different enzymes, we studied the sensitivity to the inhibitor molybdate. In agreement with previous results (18), we observed that PPase is greatly inhibited (approximately 80%) by molybdate (Fig. 2B),

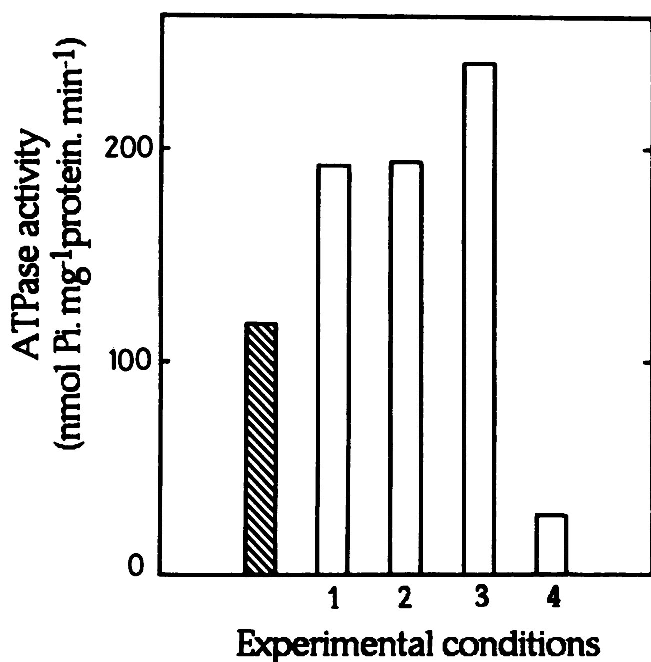

Figure 1. ATPase activity in light microsomes of corn roots under several experimental conditions. The ATPase activity was measured in a sulfate-containing medium as described in "Materials and Methods," excepting some assays in which chloride salts were used instead sulfate salts. Cross-hatched bar, ATPase activity in a chloride-containing medium; white bars, ATPase activity in a sulfate-containing medium; 1, control; 2, presence of Ca^{2+} (0.3 mM); 3, presence of Na-deoxycholate (0.01%); 4, presence of DIDS (100 μM).

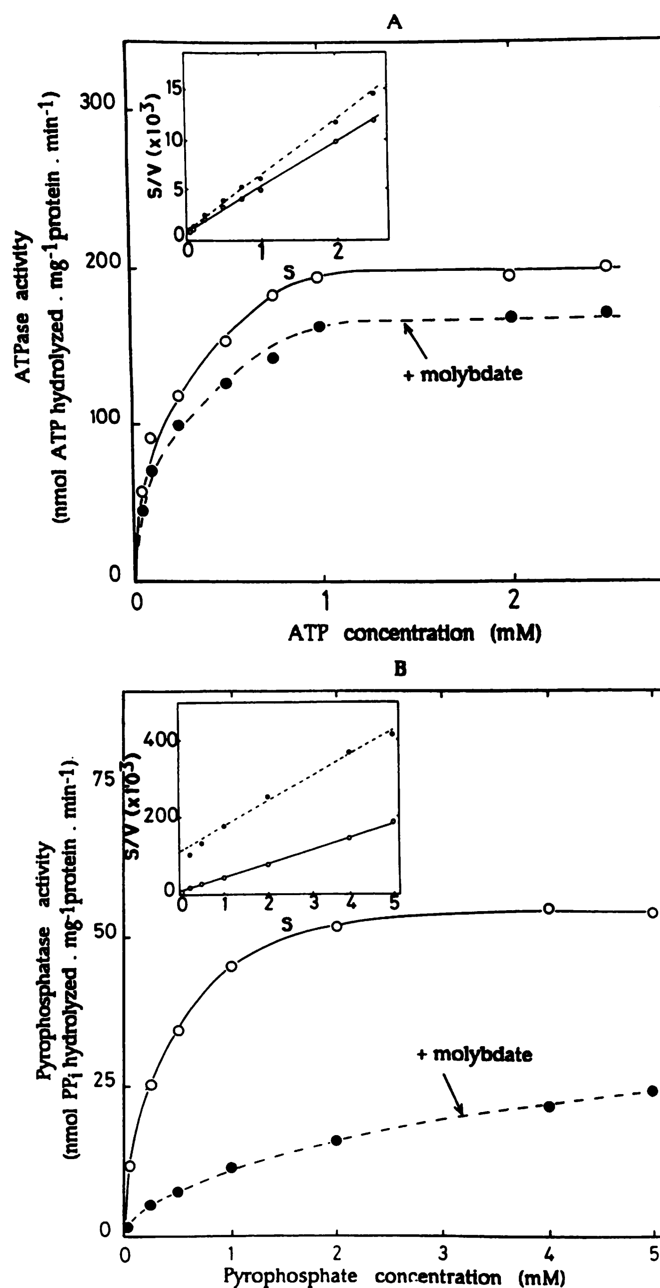

Figure 2. Phosphohydrolase activities as a function of increasing ATP-Mg and PPi concentrations. The phosphohydrolysis of ATP and PPi by light microsomes of corn roots was assayed as described in "Materials and Methods." A, ATPase activity; B, PPase activity. Inset: Hanes-Woolf analysis for the results depicted in the curves. (○) Activity in the absence of molybdate; (●) activity in the presence of molybdate (150 μM).

whereas a small inhibitory effect (approximately 20%) was observed in the case of the ATPase (Fig. 2A). Molybdate did not alter significantly the K_m (ATP) (approximately 160 μM), but the K_m (PPi) was greatly enhanced from the value 240 to 1900 μM in the presence of the inhibitor. However, PPase inhibition was not due to simple competition, because the V_{max} values of the PPase were also significantly reduced from about 57 to 34 nmol PPi hydrolyzed mg protein^{-1} min^{-1} in the presence of the inhibitor (Fig. 2B).

Because the phosphohydrolase activities of the ATPase and PPase differ in their kinetic parameters and in their sensitivity to molybdate, it is plausible to assume that light microsomes of corn roots have two distinct enzymes that hydrolyze ATP and PPi, respectively. Indeed, we observed an increased phosphohydrolase activity when saturable concentrations of both substrates, ATP and PPi, were simultaneously added to the reaction medium (Fig. 3A, c″ bar). We also observed that the activity (approximately 64 nmol Pi mg protein^{-1} min^{-1}) of the molybdate-sensitive PPase (dotted bars) remained unaltered in the presence of several concentrations of ATP (Fig. 3A, c, c′, c″ bars), whereas the molybdate-insensitive ATPase (cross-hatched bars) was inhibited by PPi (2 mM), particularly at low concentrations of ATP. At 0.5 mM ATP, PPi inhibited the ATPase by about 47% (Fig. 3A, arrow 1), whereas at 2 mM ATP, only 5% inhibition occurred (Fig. 3A, arrow 3). These findings were supported by the results depicted in Fig. 3B. In these experiments, ATPase activity was determined by measuring ^{32}Pi liberation from [^{32}P]ATP in the presence of molybdate. As in Fig. 3A, the same type of ATPase inhibition was observed in the presence of PPi.

The results indicate that ATP had no effect on the PPase activity, whereas PPi inhibited the ATPase by a process that was essentially competitive, as judged by the low inhibitory effect found at high ATP concentrations (2 mM) (Fig. 3A, B). Indeed, Hanes-Woolf analysis depicted in Figure 4 shows that, under conditions at which PPase does not work (molybdate present), PPi increased the value of K_m (ATP) from about 154 to about 500 μM, whereas the V_{max} value was not significantly altered (approximately 170 nmol Pi mg protein^{-1} min^{-1}) (Fig. 4, inset).

Because molybdate has been described as a potent inhibitor of acid phosphatase (9, 17, 31), we investigated whether the PPase activity observed here is related to this enzyme. Thus, we studied how PPase operates at pH values beyond the neutral range. Figure 5A shows that at pH 4.8 PPase was greatly reduced (approximately 65% from the maximum at neutral pH), whereas the acid phosphatase was maximal (approximately 360 nmol *p*-nitrophenylphosphate hydrolyzed mg protein^{-1} min^{-1}) (Fig. 5B). In contrast, at pH 7.0, which was optimal for the PPase (Fig. 5A), little acid phosphatase was observed (Fig. 5B). It appears, therefore, that PPase activity is distinct from the acid phosphatase. On the other hand, we observed that PPase activity (Fig. 5A), as well as acid phosphatase activity (Fig. 5B), were strongly inhibited (approximately 80%) at pH 8.5 (Fig. 5A, B). It appears that the PPase activity observed here does not correspond to the alkaline PPase (pH 8.5) reported by several investigators in tonoplast membranes (13, 25, 30–32). Indeed, by using molybdate, we could distinguish between a neutral (molybdate-sensitive) and an alkaline (molybdate-insensitive) PPase, which predominate in the F16 and F34 fractions, respectively. These findings indicate that different types of PPase enzymes

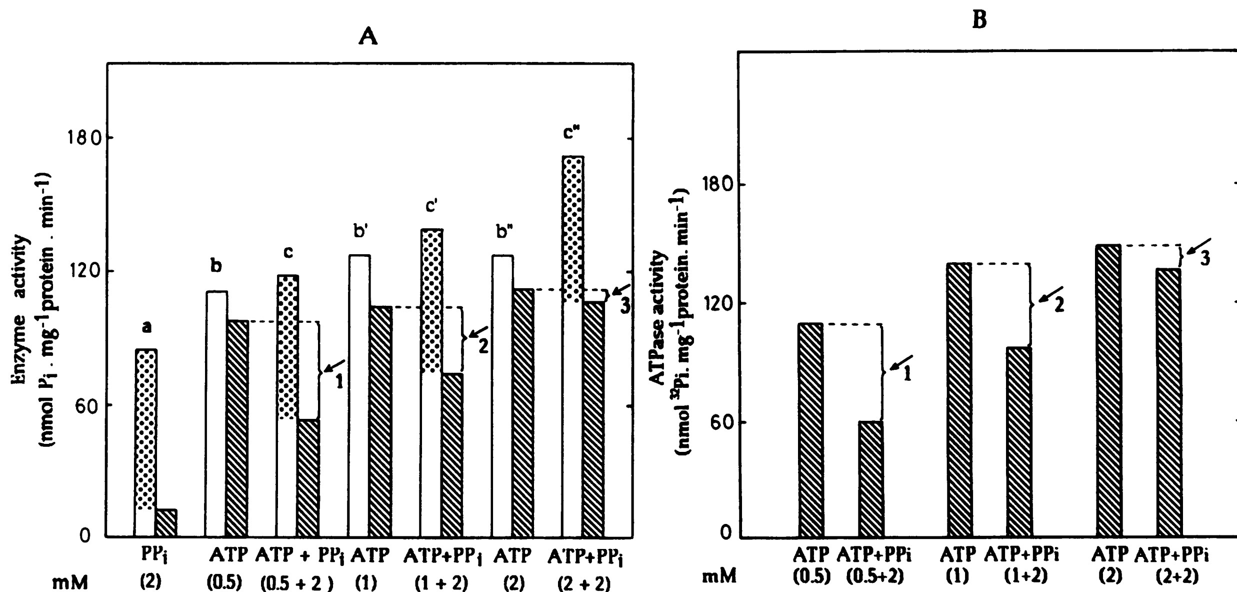

Figure 3. Effect of PPi and ATP on the activities of the ATPase and PPase, respectively. The phosphohydrolase activity of corn root microsomes was assayed in the presence of each substrate (ATP or PPi) added to separate reactions, or in the presence of both substrates added together to the reaction medium. A, Spectrophotometric analysis of Pi liberation; B, radiometric analysis of ^{32}Pi liberation. The cross-hatched bars indicate the enzyme activity in the presence of molybdate (150 μM). The dotted portions of the bars indicate PPase activity that is molybdate-sensitive. The white bars represent ATPase activity. Arrows 1, 2, and 3 represent the extent of ATPase inhibition by PPi (2 mM) in the presence of 0.5, 1, and 2 mM of ATP. The assays were performed as described in "Materials and Methods."

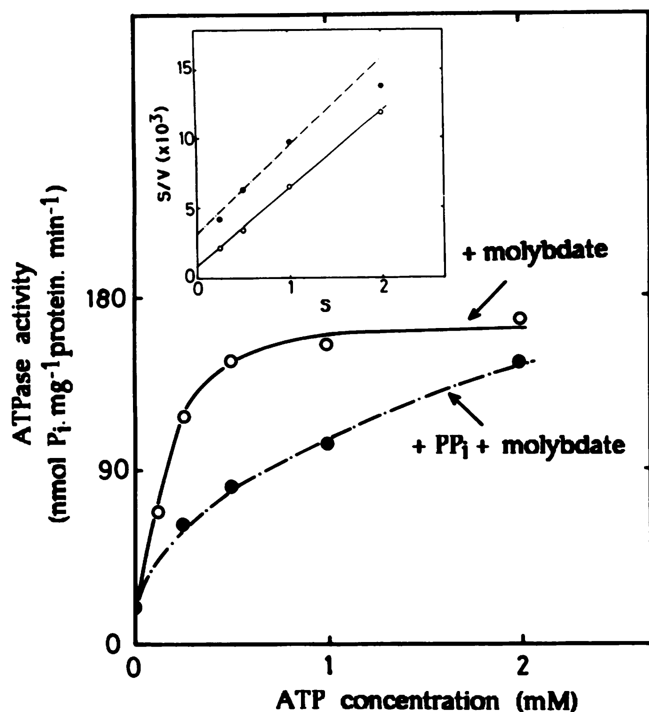

Figure 4. Characterization of the ATPase inhibition by PPi. The ATPase activity was assayed in the presence of PPi (2 mM) under conditions at which PPase is inactive (presence of 150 μM molybdate). (○) Activity in the absence of PPi; (●) activity in the presence of PPi. Inset: Hanes-Woolf analysis for the results depicted in the curves. The assays were performed as described in "Materials and Methods."

work in the tonoplast and in the light microsomes of corn roots.

Characterization of IDPase Activity in Light Microsomes of Corn Roots

Light microsomes of corn roots have a high IDPase activity that increased by storage at low temperatures (0–4°C) and by treatment with detergents, in agreement with previous investigations (14, 19, 21). Furthermore, it was not significantly sensitive to molybdate and it was not altered in the presence or absence of sulfate (results not shown).

As observed previously (21), the IDPase activity was higher in the presence of Triton X-100 than in its absence (Fig. 6). This latent activity increased as IDP concentration increased in the medium up to about 2 mM. Above this concentration, stimulation by Triton decreased up to about 5 mM IDP. It appears that when IDPase is fully active in the presence of detergent, it is saturated at low concentrations of IDP. Therefore, detergent must eliminate the enzyme activation promoted, in intact membranes, by high IDP concentrations (Fig. 6). This explains the high K_m values (approximately 700 μM) for the total IDPase activity observed in native membranes, in which maximal velocities appear only at IDP concentrations above 4 mM.

Using molybdate as an inhibitor of the PPase, we could distinguish the activity of this enzyme from that of the IDPase. Figure 7 shows that, in the presence of both substrates, PPi and IDP, the PPase activity, which is obtained by subtracting the phosphohydrolase activity in the presence of molybdate from the total activity in the absence of molybdate, was not altered by several concentrations of IDP (0.5, 1, and 4 mM) (Fig. 7, dotted bars). This indicates that, when both substrates are added together to the reaction medium, the PPase is not altered by IDP. In contrast, the IDPase activity, which is evaluated by the remaining phosphohydrolase activity observed after addition of molybdate (Fig. 7, cross-hatched bars), was lower in the presence of PPi compared with that observed in its absence (Fig. 7). The results indicate that the IDPase is inhibited by PPi, whereas the PPase is not affected by IDP.

The inhibitory effect of PPi on the IDPase depends on the concentration of IDP (Fig. 7). We observed 75% of IDPase inhibition by PPi at 0.5 mM IDP in the reaction medium, whereas only 40% inhibition was observed at 4 mM IDP. However, the effect was not due to simple competition. Lineweaver-Burk analysis showed that, under conditions at which PPase does not work (molybdate present), PPi increased the value of K_m (IDP) from about 720 to about 1380 μM, whereas the V_{max} value decreased from about 400 to 260 nmol Pi mg protein^{-1} min^{-1} (Fig. 8).

The IDPase activity of light microsomes of corn roots was also distinguished from the ATPase activity. Figure 9A shows how the presence of ATP (1 or 2.5 mM) changes the kinetic behavior of the phosphohydrolase activity as a function of IDP concentration. At IDP concentrations above 0.5 mM, we observed that 2.5 mM ATP decreased the phosphohydrolase activity (black circles) as compared to that observed in the absence of ATP (white circles). However, it remained higher (>200 nmol Pi mg protein^{-1} min^{-1}) than that (approximately 170 nmol Pi mg protein^{-1} min^{-1}) observed when only ATP, at saturating levels (2.5 mM), was present in the medium

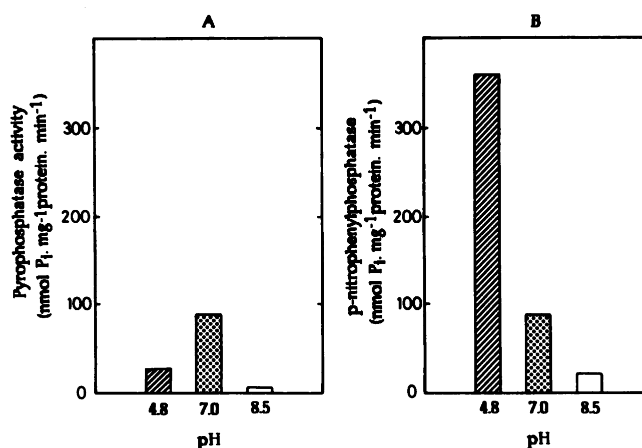

Figure 5. Differentiation between PPase and acid phosphatase activities on the basis of their optimal pH. The activities of PPase and acid phosphatase were studied at several pH values by measuring the hydrolysis of PPi (2 mM) and *p*-nitrophenylphosphate (2 mM), respectively. A, PPase activity; B, acid phosphatase activity. Three pH values were considered: 4.8 (cross-hatched bars), 7.0 (dotted bars), and 8.5 (white bars). The assays were performed as described in "Materials and Methods."

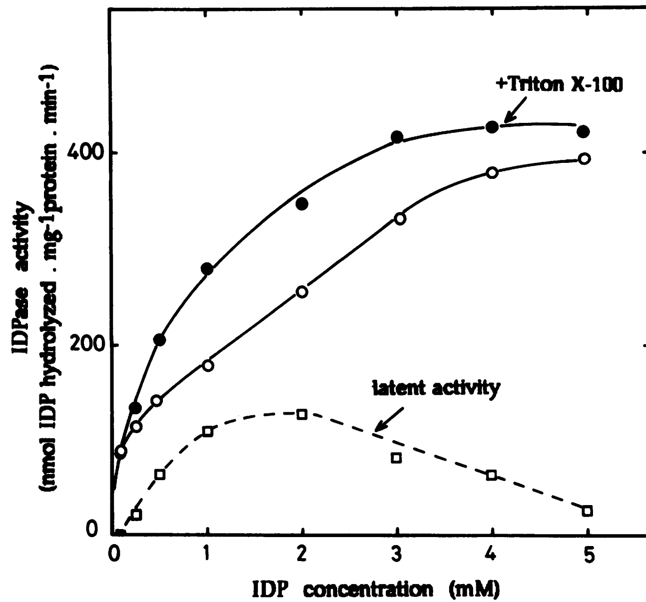

Figure 6. Effect of Triton X-100 on the IDPase activity of corn root microsomes. The IDPase activity was measured in the presence or absence of Triton X-100 (0.05%), as described in "Materials and Methods." (○) Absence of detergent; (●) presence of detergent. (□) Latent IDPase activity calculated by subtracting the IDP hydrolysis observed in the absence of detergent from that observed in its presence.

(ATPase). This suggests that two different enzymes were operative in the presence of both substrates and that ATP inhibited the IDPase. Under these conditions, no evidence could be obtained about a possible effect of IDP on the ATPase. Therefore, experiments were carried out using lower inhibitory concentrations of ATP (1 mM). In this case, we observed higher phosphohydrolase activity (Fig. 9A, broken line) compared with that observed in the absence of ATP (white circles). However, this cumulative phosphohydrolysis, which represents ATPase activity, was reduced at the highest IDP concentrations used, suggesting that IDP inhibits the ATPase (Fig. 9A). This finding was confirmed in experiments in which [^{32}P]ATP was used to distinguish the Pi liberation associated to ATPase activity from that associated to IDPase activity (Fig. 9B). Indeed, at various concentrations of ATP (0.5, 1, and 2.5 mM), we found that the ATPase is inhibited by about 20 or 35% by 1 mM IDP or 4 mM IDP, respectively. Taking into consideration these values of ATPase activity, we could calculate the contribution of the IDPase for the total phosphohydrolase activity under the various experimental conditions studied (Table I).

By comparing the IDPase activities measured in the presence of ATP with those obtained in its absence, we observed that the IDPase is partially inhibited by 1 mM ATP (32–34%) in a process that is not competitive, whereas 2.5 mM ATP has a superimposed inhibitory effect that appears to be competitive with IDP (Table I).

Membrane Characterization of Different Microsomal Fractions Isolated from Corn Roots

To identify the type of membranes obtained in the light microsomal fraction of corn roots, we assayed the activity of several membrane markers. As indicated in Table II, we observed that corn root membranes collected at the sample/16%, 16/34% and 34/40% interfaces from the discontinuous sucrose gradients (F16, F34, and F40 fractions) were enriched in endoplasmic reticulum, tonoplast, and plasma membranes, respectively. Indeed, we found higher activity of NADPH-Cyt *c* reductase (226 nmol reduced Cyt c mg protein^{-1} min^{-1}) in the F16 fraction than in the other fractions, whereas the activities of the nitrate-inhibited ATPase (20 nmol Pi mg protein^{-1} min^{-1}) and the vanadate-sensitive ATPase (66 nmol Pi mg protein^{-1} min^{-1}) were higher in the F34 and F40 fractions, respectively. This membrane distribution is compatible with that previously obtained by other investigators using step gradients of sucrose (1, 6, 22).

The nitrate inhibition of the F34 ATPase (tonoplast) was essentially visualized in the presence of vanadate, which eliminates the contribution of some contaminating plasma membrane ATPase. In contrast to F34, no inhibition by nitrate was observed in the F16, which indicates absence of tonoplast membranes in the light microsomal fraction of corn roots. This observation was supported by measurements of the molybdate-insensitive alkaline PPase, which was active only in the heavier microsomes (Table II).

In addition to enrichment in endoplasmic reticulum, F16 also contained Golgi membranes as revealed by the latent IDPase activity (290 nmol Pi mg protein^{-1} min^{-1}), which was higher in the F16 than in the F34 and F40 fractions (Table II). Furthermore, F16 is also characterized by a sulfate-stimulated ATPase, which was negligible in the other fractions (Table II). These observations indicate that different types of membranes predominate in the various microsomal fractions isolated from corn roots. The F16 was the most purified

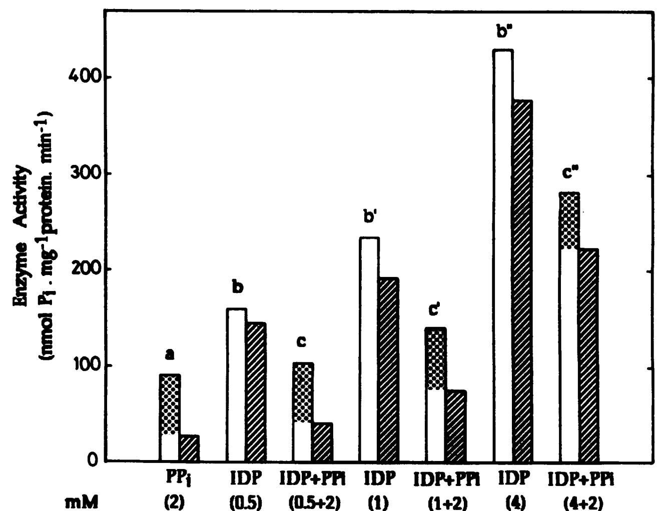

Figure 7. Effect of PPi and IDP on the activities of the IDPase and PPase, respectively. The phosphohydrolase activity was assayed in the presence of each substrate (IDP and PPi) added to separate media, or in the presence of both substrates added together to the reaction medium. The cross-hatched bars indicate the enzyme activity in the presence of molybdate (150 μM). The dotted portions of the bars indicate PPase activity that is molybdate-sensitive. The white bars represent IDPase activity. The assays were performed as described in "Materials and Methods."

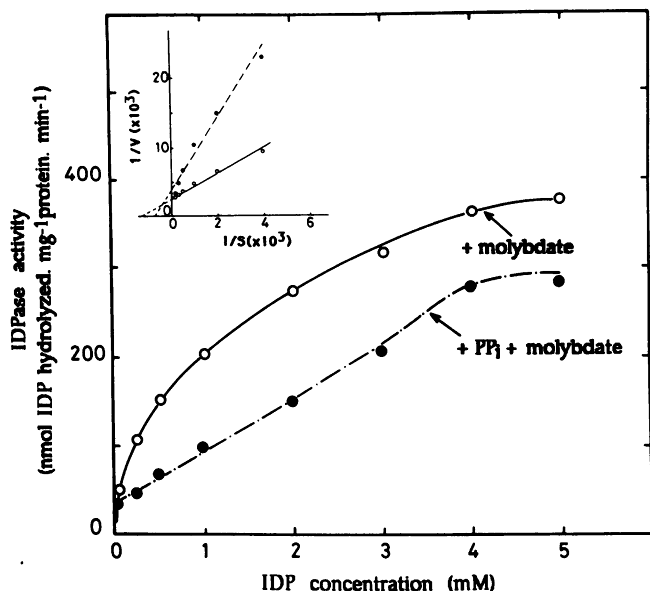

Figure 8. Characterization of the IDPase inhibition by PPi. The IDPase activity was assayed in the presence of PPi (2.5 mM) under conditions at which PPase is inactive (presence of 150 μM molybdate). (○) Activity in the absence of PPi; (●) activity in the presence of PPi. Inset: Lineweaver-Burk analysis for the results depicted in the curves. The assays were performed as described in "Materials and Methods."

fraction, whereas the other fractions showed some contaminating endoplasmic reticulum membranes. It appears that discontinuous gradients are suitable for isolation of endoplasmic reticulum and Golgi membranes, whereas tonoplast and plasma membranes are better purified in continuous gradients of sucrose.

DISCUSSION

We have characterized the phosphohydrolase activity in a low-density membrane fraction of corn roots that appears to be enriched in endoplasmic reticulum and Golgi membranes. The light microsomal membranes of corn roots contain distinguishable phosphohydrolases that hydrolyze PPi, ATP, and IDP.

The ATPase and PPase activities differ in their kinetic parameters and sensitivity to molybdate. They represent activities of separate enzymes, as was demonstrated by the fact that phosphohydrolysis increased when ATP and PPi were simultaneously added to the reaction medium, even at saturating concentrations. On the other hand, we observed maximal PPase activity at neutral pH (Fig. 5A), indicating that phosphohydrolysis does not derive from acid phosphatase activity, which works at pH 4.8 (Fig. 5B).

PPase activity has been reported to be associated with vacuolar membranes (4, 5, 25, 26, 30–32). However, the microsomal PPase observed here appears to be distinct from the tonoplast PPase, because it works maximally at pH 7.0 and is sensitive to molybdate, whereas the tonoplast PPase is molybdate-insensitive and it has an optimum pH of 8.5 (13, 25, 30–32).

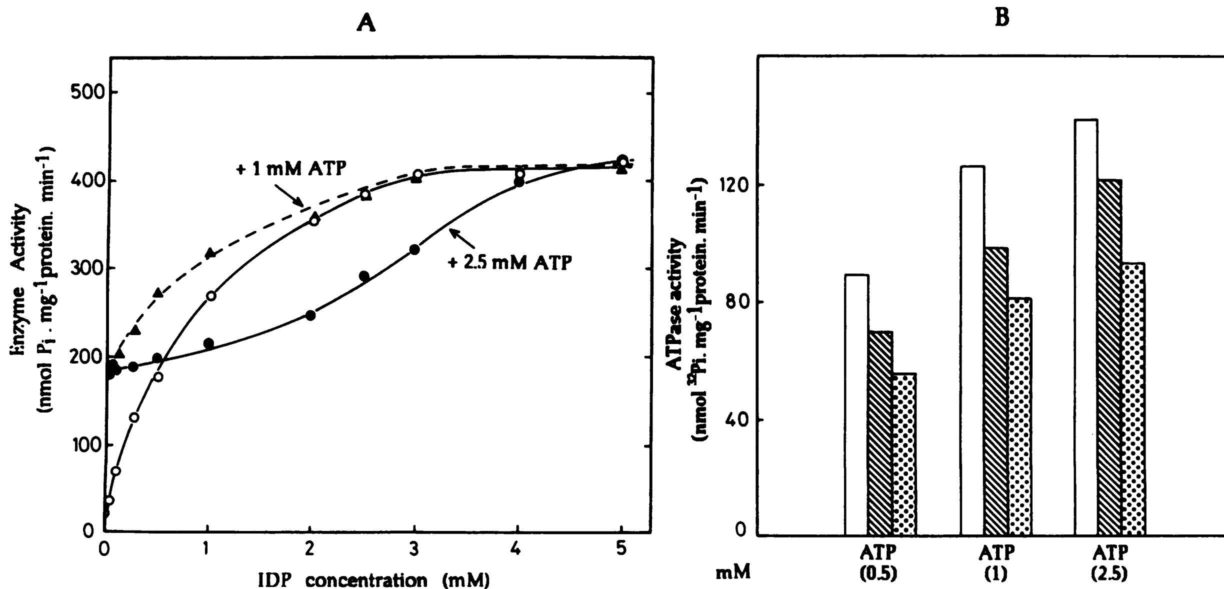

Figure 9. Differentiation between the effects of ATP on the IDPase and the effect of IDP on the ATPase. A, Phosphohydrolase activity of corn root microsomes measured spectrophotometrically as a function of IDP concentration in the absence or in the presence of ATP. (○) IDPase activity in the absence of ATP; (▲) phosphohydrolase activity in the presence of 1 mM ATP; (●) phosphohydrolase activity in the presence of 2.5 mM ATP. B, ATPase activity radiometrically assayed by measuring ^{32}Pi liberation from [^{32}P]ATP. White bars, absence of IDP; cross-hatched bars, presence of 1 mM IDP; dotted bars, presence of 4 mM IDP. The assays were performed as described in "Materials and Methods."

Table I. *Effect of ATP on the IDPase Activity*

The values depicted in the table represent IDPase activity that was calculated from the results shown in Figure 8A and B. The IDPase activity was determined by subtracting the ATPase activity (*^{32}Pi liberation) from the total phosphohydrolase activity (Pi liberation) under variable conditions of ATP and IDP concentrations.*

ATP	IDPase Activity	
	1 mM IDP	4 mM IDP
mM	*nmol P_i*	*$mg^{-1}min^{-1}$*
0	260	400
1	176 (32%)[a]	263 (34%)
2.5	66 (75%)	263 (34%)

[a] The values within parentheses are the percentages of IDPase inhibition by 1 or 2.5 mM ATP.

The physiological role of the membrane-bound PPases is still not understood. There is no doubt that ATP is the most important energy donor for the energization of transport across plant cell membranes. However, the involvement of other phosphoesters such as PPi has been recently demonstrated by several investigators (4, 18, 25, 32).

Although the coupling reactions between PPi hydrolysis and H^+ transport are still not known, it appears that plant cell membranes contain an H^+-pumping PPase, which is functionally distinct from the ATPase (26, 30, 32). Our results support this concept and show that PPi inhibits the ATPase (Figs. 3, 4).

We also differentiated the IDPase as an enzyme distinct from the other phosphohydrolases. This IDPase was stimulated by Triton X-100 (Fig. 6), but to a lower extent than that reported by other investigators (21). Probably, the enzyme was somewhat activated by previous cold storage (4 d), which, like detergents, stimulates IDPase activity (14, 19).

Interestingly, we found a high K_m value (700 μM) for the native IDPase activity, whereas the latent IDPase had a lower K_m value. Furthermore, no increased activity was observed at high concentrations of IDP (>3 mM) in the presence of Triton X-100 (Fig. 6), which indicates that all enzyme molecules were made fully active by detergent and lost their capacity to be activated by high IDP concentrations. Indeed, the action of Triton in exposing the enzyme has been demonstrated by increased trypsin attack (20). These results suggest that the IDPase enzyme contains two distinct binding sites for IDP: a catalytic site with high affinity (K_m < 200 μM) and a regulatory site with low affinity. In native membranes, the enzyme appears to be repressed when the regulatory site is not occupied by IDP, suggesting that IDP is a positive modulator of the enzyme. The repressor site does not work in detergent-attacked enzyme, so that it is fully active at lower IDP concentrations.

These conclusions are supported by the results obtained in the presence of PPi and ATP, which inhibit the IDPase by a process that is not due to simple competition (Figs. 8, 9A). It appears that PPi and ATP act on the regulatory site of the IDPase, behaving as negative modulators of the enzyme. This site has low affinity for its ligands, because it is sensitive to high concentrations of IDP (>2 mM) and ATP (2.5 mM). As is shown in Table I, 1 mM ATP partially inhibits the IDPase in a manner that is not competitive with IDP, whereas 2.5 mM ATP has a strong inhibitory effect that reflects competition with IDP. It appears that ATP interacts with two sites of the enzyme, but only that of lower affinity serves modulation of the IDPase by ATP and IDP. In contrast to IDPase, PPase is not altered by either ATP or IDP, which indicates that it is not a nucleotide-regulated enzyme. On the other hand, the ATPase appears to be competitively inhibited by PPi (Fig. 4) and noncompetitively inhibited by IDP (Fig. 9B).

Cytoplasmic PPi is formed from anabolic reactions that occur at high rates during growth of tissues. Under these conditions, PPase activity shifts the equilibrium of reactions in favor of net biosynthesis. Furthermore, the energy of PPi hydrolysis may be utilized to drive proton transport, which probably reduces ATP consumption in membrane transport processes, as suggested by Chanson *et al.* (4). The neutral PPase reported here appears to be different from the alkaline proton-pumping PPase that has been found in tonoplast membranes (13, 25, 30–32). They differ in their optimum pH value, in their sensitivity to molybdate, and in their distribution in sucrose gradients.

With respect to IDPase activity, it has been reported as a Golgi marker enzyme (24). Its function is not well established, but it appears to be involved in polysaccharide synthesis (10), where an NDPase-catalyzed reaction is coupled to a glycosyl transferase-catalyzed reaction (15). Our results indicate that

Table II. *Relative Distribution of the Activities of Membrane Marker Enzymes in Three Fractions Obtained from a Step Sucrose Gradient*

Microsomal vesicles of corn roots were separated with a three-step sucrose gradient. Interfaces from sample/16% (F16), 16/34% (F34), and 34/40% (F40) were collected and the marker enzyme activities measured as described in the "Materials and Methods."

Fractions	Enzyme Activity						
	Vanadate-inhibited ATPase	Nitrate-inhibited ATPase[a]	Molybdate-insensitive PPase	Molybdate-inhibited PPase	Latent IDPase	Sulfate-stimulated ATPAse	NADPH-Cyt *c* reductase
	nmolPi mg protein^{-1} min^{-1}						*nmol reduced Cytc mg protein^{-1} min^{-1}*
F16	20	0	10	84	290	90	226
F34	50	20	57	22	94	2	80
F40	66	0	38	19	78	0	100

[a] The activity expressed for the nitrate-inhibited ATPase represents the sensitivity to nitrate in the presence of 50 μM vanadate.

native IDPase has a regulatory site that is positively modulated by IDP and negatively modulated by PPi and ATP.

Additional studies are currently in progress in our laboratory to investigate the function and regulation of all these enzymes.

ACKNOWLEDGMENTS

We are grateful to the Institute of Botany of the University of Coimbra for providing the laboratory space and technical assistance during the germination of corn seeds. We also thank the Agricultural Association of Coimbra for supplying the corn seeds.

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