Plant Physiol. (1985) 78, 495–499
0032-0889/85/78/0495/05/$01.00/0

H^+-ATPase Activity from Storage Tissue of *Beta vulgaris*[1]

III. MODULATION OF ATPase ACTIVITY BY REACTION SUBSTRATES AND PRODUCTS

Received for publication September 24, 1984 and in revised form March 15, 1985

ALAN B. BENNETT*[2], SHARMAN D. O'NEILL[3], MARIANNE EILMANN[4], AND ROGER M. SPANSWICK
Section of Plant Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14853

ABSTRACT

Two distinct membrane fractions containing H^+-ATPase activity were prepared from red beet. One fraction contained a H^+-ATPase activity that was inhibited by NO_3^- while the other contained a H^+-ATPase inhibited by vanadate. We have previously proposed that these H^+-ATPases are associated with tonoplast (NO_3^--sensitive) and plasma membrane (vanadate-sensitive), respectively. Both ATPases were examined to determine to what extent their activity was influenced by variations in the concentration of ATPase substrates and products. The substrate for both ATPases was $MgATP^{2-}$, and Mg^{2+} concentrations in excess of ATP had only a slight inhibitory effect on either ATPase. Both ATPases were inhibited by free ATP (*i.e.* ATP concentrations in excess of Mg^{2+}) and ADP but not by AMP. The plasma membrane ATPase was more sensitive than the tonoplast ATPase to free ATP and the tonoplast ATPase was more sensitive than the plasma membrane ATPase to ADP.

Inhibition of both ATPases by free ATP was complex. Inhibition of the plasma membrane ATPase by ADP was competitive whereas the tonoplast ATPase demonstrated a sigmoidal dependence on $MgATP^{2-}$ in the presence of ADP. Inorganic phosphate moderately inhibited both ATPases in a noncompetitive manner.

Calcium inhibited the plasma membrane but not the tonoplast ATPase, apparently by a direct interaction with the ATPase rather than by disrupting the $MgATP^{2-}$ complex.

The sensitivity of both ATPases to ADP suggests that under conditions of restricted energy supply H^+-ATPase activity may be reduced by increases in ADP levels rather than by decreases in ATP levels *per se*. The sensitivity of both ATPases to ADP and free ATP suggests that modulation of cytoplasmic Mg^{2+} could modulate ATPase activity at both the tonoplast and plasma membrane.

Electrogenic H^+ transport is thought to be the primary transport event occurring across the plasma membrane and tonoplast of plant cells. Proton-translocating ATPases have been identified in both isolated plasma membrane and tonoplast vesicles of red beet (5). These H^+-ATPases are most likely responsible for the majority of active H^+ transport across these membranes. Early attempts to relate the activity of electrogenic H^+ transport across the plasma membrane to levels of cytoplasmic ATP were only partially successful (13). While experiments indicated that ATP is indeed the source of energy for H^+ transport, the lack of complete correspondence between ATP levels and electrogenic H^+ transport activity suggested that regulatory mechanisms other than energy supply (*i.e.* ATP levels) were operative in controlling H^+ transport (13). Similar conclusions were also inferred regarding the activity of H^+ transport across the tonoplast, since the observed gradients of $\Delta\tilde{\mu}_{H^+}$ *in vivo* are generally far from the thermodynamic equilibrium predicted for a H^+-ATPase with a H^+/ATP coupling stoichiometry of 2 (6).

Although it now seems apparent that H^+ transport is not regulated by energy supply *per se*, it is likely that H^+-ATPase activity would be responsive to some component(s) of the cellular energy charge since these ATPases may account for a significant proportion of the turnover of cellular ATP. In order to begin to assess the influence of cellular constituents which may contribute to regulation of plasma membrane and tonoplast H^+-ATPase activity *in vivo*, we have examined the effects of reaction substrates and products on H^+-ATPase activity associated with isolated plasma membrane and tonoplast vesicles from red beet.

MATERIALS AND METHODS

Membrane Preparation. Membranes were prepared from the storage tissue of red beet as previously described (5). Fractions enriched in NO_3^--sensitive and vanadate-sensitive ATPase activity were collected from the 16/26% and 34/40% interfaces, respectively, of a discontinuous sucrose gradient. Approximately 80 to 90% of the ATP hydrolyzing activity in these two fractions is attributable to the activity of H^+-translocating ATPases which we propose are of tonoplast (NO_3^--sensitive ATPase) or plasma membrane (vanadate-sensitive ATPase) origin (5).

ATPase Assay. ATPase activity was measured at 25°C for 30 min with 1 to 10 μg protein per assay. The reaction was carried out in a volume of 0.5 ml containing 50 mM KCl, 30 mM Tris/Mes, 2 μM gramicidin, with concentrations of Tris-ATP, $MgSO_4$, and other additions as indicated in the text or figure legends. Phosphate was determined by the method of Ames (2). Reaction blanks (*i.e.* no enzyme) were prepared for each treatment and subtracted to calculate the ATPase activity.

High concentrations of ATP_f[5] interfered with color development using the Ames reagent. In the presence of high concentrations of ATP_f and in the presence of Pi, ATPase activity was measured by coupling ATP hydrolysis to NADH oxidation with P-enolpyruvate/pyruvate kinase and lactate dehydrogenase and monitoring NADH oxidation spectrophotometrically (A_{340}) (6). The reaction was carried out in a volume of 1.0 ml containing 50 mM KCl, 30 mM Tris/Mes, 5 mM $MgSO_4$, 0.42 mM P-enolpyruvate, 0.15 mM NADH, 12 units lactate dehydrogenase, and 7 units pyruvate kinase. Neither high concentrations of ATP_f

[1] Supported by United States Department of Agriculture Competitive Grants Program grant 81-CRCR-1-0758 and National Science Foundation grant PCM 81-11007.

[2] Present address: Mann Laboratory, Department of Vegetable Crops, University of California, Davis, CA 95616.

[3] Present address: Plant Growth Laboratory, University of California, Davis, CA 95616.

[4] Present address: Advanced Genetic Sciences, Inc., 6701 San Pablo Avenue, Oakland, CA 94608.

[5] Abbreviation: ATP_f, free ATP.

nor Pi interfered with reactions coupling ADP production to NADH oxidation.

The assay pH was adjusted to 6.5 for plasma membrane fractions and 7.0 for tonoplast fractions.

$MgATP^{2-}$, Free ATP, Free Mg^{2+} Calculations. $MgATP^{2-}$, free Mg^{2+}, and ATP_f(ATP^{4-} + $HATP^{3-}$ + H_2ATP^{2-}) were calculated using the iterative procedure of Storer and Cornish-Bowden (14) using association constants published by Alberty (1).

RESULTS

Both plasma membrane and tonoplast ATPase activities have been shown to be Mg^{2+} dependent (3, 16). Figure 1 shows the concentration dependence of both plasma membrane and tonoplast ATPases (Fig. 1A) for Mg^{2+} when assayed in the presence of 5 mM ATP. The results in Figure 1 are similar to those of Balke and Hodges (3), and Walker and Leigh (16) for plasma membrane and tonoplast ATPases, respectively. These results suggest that $MgATP^{2-}$ is the substrate for both ATPases and further indicate that increasing concentrations of excess Mg^{2+} have only a slight inhibitory effect on ATPase activity. This slight inhibition by high Mg^{2+} may result from the formation of Mg_2ATP, and consequent reduction in $MgATP^{2-}$ concentration (15). When Mg^{2+} was held constant and the concentration dependence of both plasma membrane and tonoplast ATPases for ATP was determined, a strikingly different result was observed (Fig. 2, A and B). At concentrations of ATP exceeding the fixed Mg^{2+} concentration (either 2.5 or 5.0 mM), ATP was strongly inhibitory. Since ATP did not inhibit ATPase activity at concentrations equal to or below the fixed concentration of Mg^{2+}, it seemed likely that the inhibitory species was uncomplexed, or ATP_f. A similar result was previously reported for the plasma membrane ATPase of oat roots (3); however, a lack of sensitivity to ATP has been reported for the sugarcane tonoplast ATPase (15). The inhibition of both ATPases reported here does not result from an effect of ATP on our determination of Pi, since these ATPase assays were performed using an enzymically coupled assay ("Materials and Methods") that measures the production of ADP and is not effected by ATP. Concentrations of ATP_f were calculated at each concentration of ATP and Mg^{2+} and the

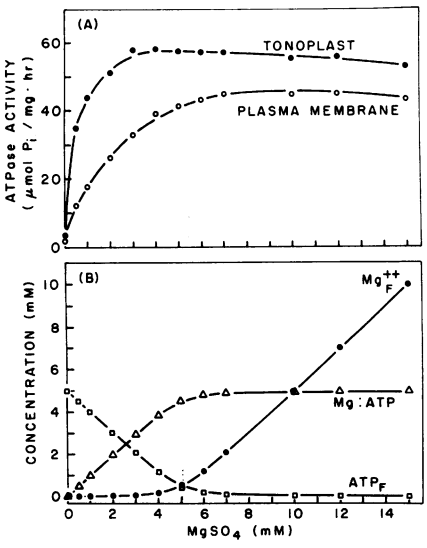

FIG. 1. A, ATPase activity of both tonoplast and plasma membrane ATPases measured in the presence of 5 mM ATP and increasing concentrations of $MgSO_4$. ATPase activity was assayed by monitoring the release of Pi as described in "Materials and Methods". B, Concentrations of free Mg^{2+} (Mg^{2+}_f), $MgATP^{2-}$, and free ATP (ATP_f = ATP^{4-} + $HATP^{3-}$ + H_2ATP^{2-}) at the indicated concentrations of $MgSO_4$ and 5 mM ATP.

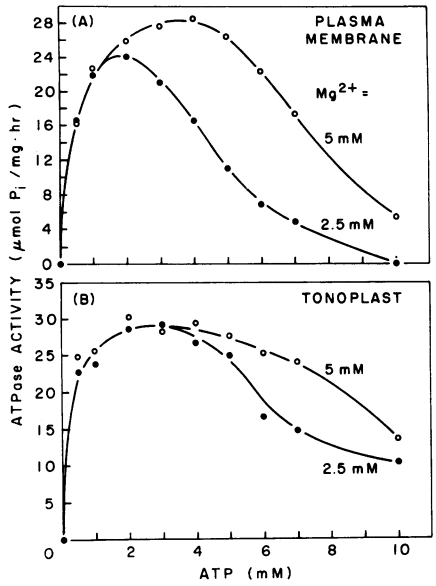

FIG. 2. ATPase activity of both plasma membrane (A) and tonoplast (B) ATPases measured in the presence of 2.5 or 5 mM $MgSO_4$ and increasing concentrations of ATP. ATPase activity was assayed by coupling ATP hydrolysis to NADH oxidation as described in "Materials and Methods".

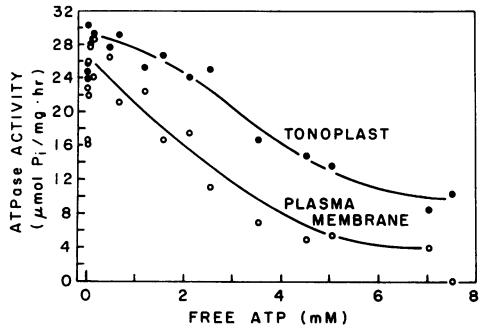

FIG. 3. ATPase activity of both tonoplast and plasma membrane ATPases as a function of free ATP. Data is replotted from Figure 2 after calculating the concentration of free ATP at each concentration of $MgSO_4$ and ATP.

data in Figure 2, A and B, replotted to show the concentration dependence of ATPase inhibition by ATP_f (Fig. 3). It is apparent from this plot of the data that the plasma membrane ATPase was more sensitive to ATP_f than was the tonoplast ATPase. The concentration of ATP_f giving half-maximal inhibition (I_{50}) was 2.1 and 4.5 mM for the plasma membrane and tonoplast ATPase, respectively. To evaluate the mechanism of ATPase inhibition by ATP_f, the MgATP concentration dependence for both ATPases was determined in the presence of increasing ATP_f (Fig. 4). The plasma membrane ATPase was inhibited by ATP_f at all MgATP concentrations (Fig. 4A). Although the effect of ATP_f on the tonoplast ATPase was less pronounced, ATP_f apparently stimulated ATPase activity at low concentrations of MgATP and inhibited activity at high MgATP concentrations (Fig. 4C). Hanes-Woolf plots of the data in Figure 4A (Fig. 4B) and Figure 4C (Fig. 4D) indicated that inhibition of ATPase activity by ATP_f was complex. A differential effect of ATP_f on the plasma membrane and tonoplast ATPase was apparent in that the K_m for MgATP increased with increasing ATP_f for the plasma membrane ATPase, but decreased with increasing ATP_f for the tonoplast ATPase. (Fig. 4, B and D).

Since it was apparent that ATP_f was much more inhibitory

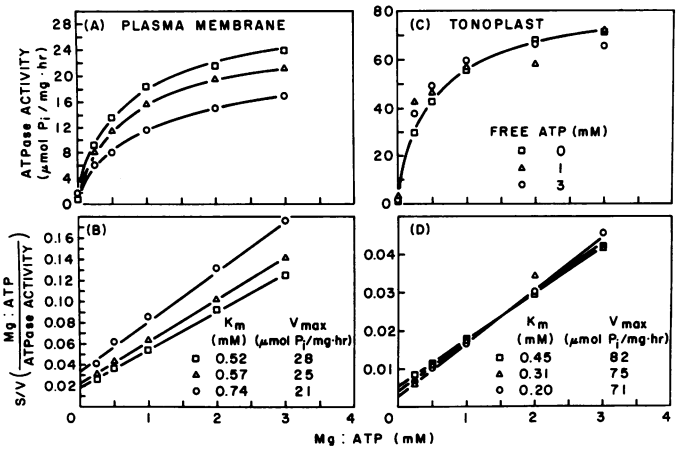

FIG. 4. MgATP concentration dependence of both plasma membrane (A and B) and tonoplast (C and D) ATPases at increasing concentrations of free ATP. ATPase activity was assayed by coupling ATP hydrolysis to NADH oxidation as described in "Materials and Methods". (B) and (D) show Hanes-Woolf plots of the data in (A) and (C).

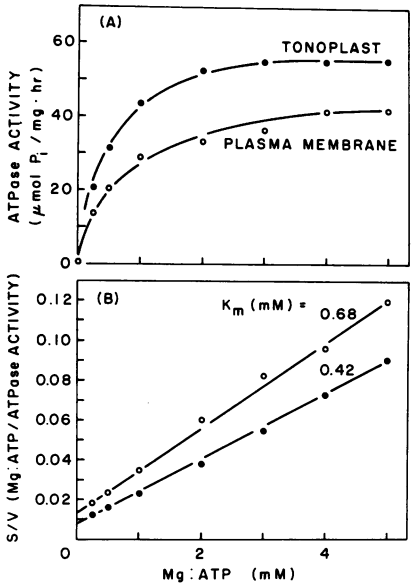

FIG. 5. MgATP concentration dependence of both tonoplast and plasma membrane ATPases assayed in the presence of a constant 2-mM excess Mg^{2+}. ATPase activity was assayed by monitoring the release of Pi as described in "Materials and Methods".

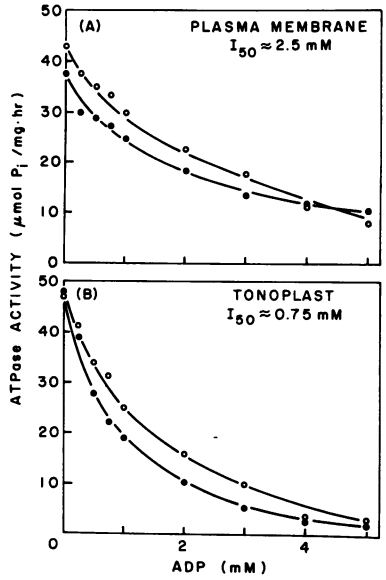

FIG. 6. ATPase activity of both plasma membrane (A) and tonoplast (B) ATPases in the presence of increasing concentrations of ADP. ATPase activity was measured in the presence of 5 mM ATP and either 5 mM (●) or 10 mM (○) $MgSO_4$. ATPase activity was assayed by monitoring the release of Pi as described in "Materials and Methods".

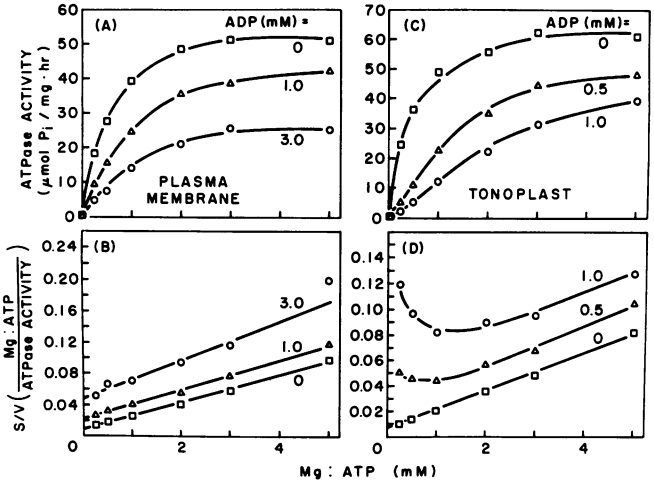

FIG. 7. MgATP concentration dependence of both plasma membrane (A and B) and tonoplast (C and D) ATPases at increasing concentrations of ADP. ATPase activity was assayed by monitoring the release of inorganic phosphate as described in "Materials and Methods". (B) and (D) show Hanes-Woolf plots of the data in (A) and (C).

than free Mg^{2+} for both ATPases, the concentration dependence for MgATP was determined by increasing MgATP concentration in the presence of a constant 2 mM excess of Mg^{2+} (Fig. 5). When assayed under these conditions, both ATPases showed simple hyperbolic kinetics with a K_m of 0.68 and 0.42 mM for the plasma membrane and tonoplast ATPase, respectively. In other experiments the K_m for the plasma membrane ATPase ranged from 0.56 to 0.83 mM and the K_m for the tonoplast ATPase from 0.42 to 0.58 mM. In general, the affinity of both ATPases for MgATP was similar enough that this characteristic is not useful in distinguishing these H^+-ATPases; nor does it suggest that these ATPases would show a differential response to reductions in MgATP concentrations *in vivo*.

Since reductions in ATP levels *in vivo* may be accompanied by increases in ADP levels (10), we examined the effect of ADP on the activity of both plasma membrane and tonoplast ATPases (Fig. 6). Because inhibition of ATPase activity by ADP could result from ADP chelation of Mg^{2+} and consequent production of ATP_f, the effects of ADP were assayed in the presence of equimolar ATP and Mg^{2+} and in the presence of 5 mM excess Mg^{2+}. The inhibition of both ATPases by ADP was similar in the presence and absence of excess Mg^{2+}, suggesting that ADP directly inhibited ATPase activity, rather than having an indirect effect on the MgATP complex. In contrast to the inhibitory effects of ATP_f, the tonoplast ATPase was much more sensitive to ADP than was the plasma membrane ATPase (Fig. 6, A and B).

The MgATP concentration dependence for both ATPases was determined in the presence of increasing concentrations of ADP (Fig. 7). The inhibitory effect of ADP on the plasma membrane ATPase appeared to be largely competitive (Fig. 7, A and B), whereas the inhibitory effect of ADP on the tonoplast ATPase was complex (Fig. 7, C and D). In the presence of ADP, the tonoplast ATPase showed a sigmoidal concentration dependence

on MgATP. This type of inhibition is observed when the inhibitor interacts with the substrate (11) and suggests that under these assay conditions, at low MgATP concentration, ADP is disrupting the MgATP complex and indirectly inhibiting ATPase activity. At higher concentrations of MgATP where ADP effects on MgATP levels are minimized, inhibition by ADP appears to be competitive (Fig. 7D) as seen for the plasma membrane ATPase (Fig. 7B). Competitive inhibition of the *Neurospora* plasma membrane ATPase by ADP (7) and depolarization of the membrane potential of internally perfused *Chara* cells (12) has been previously reported.

The effects of AMP and Pi on ATPase activities were also examined (Figs. 8, 9). AMP only slightly inhibited the plasma membrane and tonoplast ATPase at concentrations up to 5 mM with the maximum inhibition being less than 10% (Fig. 8). Pi was slightly more inhibitory than AMP with maximum inhibition being 37 and 25% for the plasma membrane and tonoplast ATPase, respectively (Fig. 9).

The MgATP concentration dependence for both ATPases was determined in the presence of 0 and 20 mM Pi (Fig. 10). Hanes-Woolf plots of the data (Fig. 10, B and C) indicated that inhibition of ATPase activity by Pi was largely noncompetitive.

Since divalent cations other than Mg^{2+} may interfere with formation of the MgATP complex, we also examined the effect of Ca^{2+} on the activity of each ATPase (Fig. 11). The plasma membrane ATPase was sensitive to inhibition by Ca^{2+} with greater than 50% inhibition observed at 1 mM Ca^{2+} (Fig. 11A); similar inhibition of the oat root plasma membrane ATPase has been reported (4). By contrast, the tonoplast ATPase was largely unaffected by Ca^{2+} with a slight, but reproducible, stimulation of ATPase activity at low concentrations of Ca^{2+} (Fig. 11B). This slight stimulation of activity is similar to that observed by DuPont *et al.* (9) in corn root microsomal vesicles. Since the assays performed here were in the presence of gramicidin, this stimulatory effect of Ca^{2+} cannot be related to Ca^{2+}/H^+ exchange affecting pH gradients but must be due to a direct interaction with the ATPase. Since Ca^{2+} can exert effects on the MgATP complex, assays were conducted in the presence of equimolar Mg^{2+}, and ATP (5 mM each) or in the presence of excess Mg^{2+} (10 mM $MgSO_4$, 5 mM ATP). In the presence of excess Mg^{2+} the MgATP complex should be stabilized against interactions with Ca^{2+} (*i.e.* formation of CaATP), yet similar results were obtained in terms of Ca^{2+} inhibition of activity. This result indicates the effects of Ca^{2+} are not related to interactions with MgATP but result from direct interactions with the ATPases.

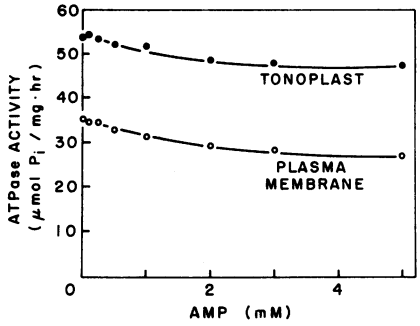

FIG. 8. ATPase activity of both plasma membrane and tonoplast ATPases in the presence of increasing concentrations of AMP. ATPase activity was assayed with 5 mM ATP and $MgSO_4$ by monitoring the release of Pi as described in "Materials and Methods".

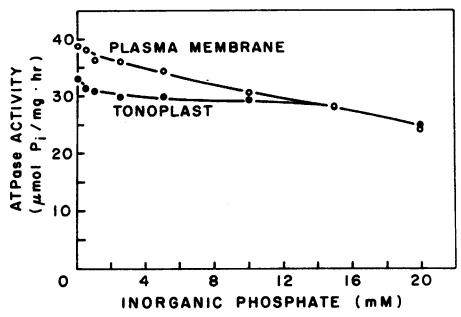

FIG. 9. ATPase activity of both plasma membrane and tonoplast ATPases in the presence of increasing concentrations of Pi. ATPase activity was assayed by coupling ATP hydrolysis to NADH oxidation as described in "Materials and Methods".

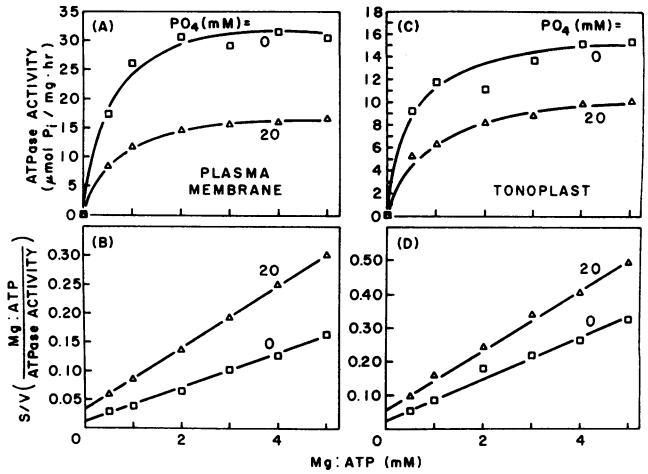

FIG. 10. MgATP concentration dependence of both plasma membrane (A and B) and tonoplast (C and D) ATPases in the presence of 0 or 20 mM Pi. ATPase activity was assayed by coupling ATP hydrolysis to NADH oxidation as described in "Materials and Methods". (B) and (D) show Hanes-Woolf plots of the data in (A) and (C).

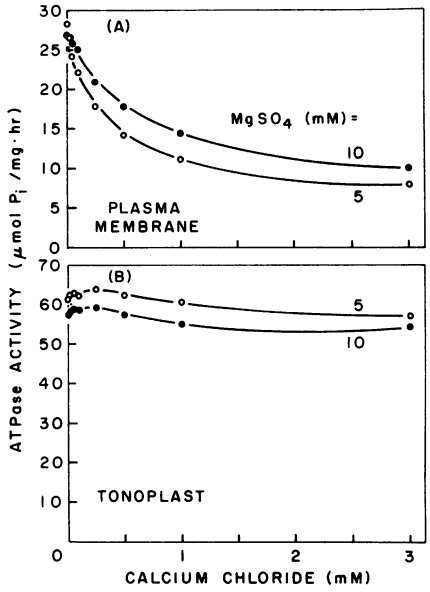

FIG. 11. ATPase activity of both plasma membrane (A) and tonoplast (B) ATPases in the presence of increasing concentrations of Ca^{2+}. ATPase activity was measured in the presence of 5 mM ATP and either 5 mM (○) or 10 mM (●) $MgSO_4$. ATPase activity was assayed by monitoring the release of Pi as described in "Materials and Methods".

DISCUSSION

As shown previously (3, 8, 16) these experiments confirm that the substrate for both plasma membrane and tonoplast ATPases

is MgATP. Both ATPases are inhibited by free ATP and by ADP, although the sensitivity of the two ATPases to free ATP and ADP differs. The plasma membrane ATPase is more sensitive to free ATP than is the tonoplast ATPase and, conversely, the tonoplast ATPase is more sensitive to ADP than is the plasma membrane ATPase. Inhibition of a plasma membrane ATPase from oat roots by free ATP has been reported (3). In this previous work, however, ATPase activity was measured by a colorimetric determination of liberated phosphate. As we, and others (15), have noted, high concentrations of free ATP interfere with formation of the colored phosphomolybdate complex. The results presented here were obtained by measuring ATPase activity in an enzyme linked assay that is not affected by free ATP. Consequently, we feel the results presented here demonstrate direct inhibition of both ATPases by free ATP.

The sensitivity of both ATPases to ADP suggests that ADP could play a role in modulating ATPase activity *in vivo*. Under conditions of high energy charge, ADP levels are quite low *in vivo* but increase in correspondence with decreasing ATP levels under conditions of diminished energy supply (10). Inhibition of H^+ transport activity under conditions of energy deprivation may occur as a direct result of increases in ADP, rather than from decreases in ATP. Consequently, attempts to correlate H^+ transport activity to ATP levels alone may be confounded by changes in other components of the cellular energy charge.

The membrane potential of internally perfused *Chara* cells has been shown to be a function of both ATP and ADP concentrations, with ATP hyperpolarizing and ADP depolarizing the membrane potential (12). These results suggest that the effects of ADP on ATPase activity measured *in vitro* are also manifested *in vivo* and may play an important role in modulating H^+ transport activity across the plasma membrane and tonoplast.

The plasma membrane ATPase was sensitive to inhibition by calcium but the tonoplast ATPase was not. Previous observations of Ca^{2+} inhibition of plasma membrane ATPase activity have been attributed to interference with the enzyme-substrate complex. The experiments performed here, in the presence of excess Mg^{2+}, would minimize effects of Ca^{2+} on MgATP levels and indicate that Ca^{2+} directly inhibits the ATPase. The differential sensitivity of the two ATPases to Ca^{2+} is one distinguishing characteristic of these ATPases, yet the concentrations of Ca^{2+} (>0.1 mM) required for inhibition are quite high and unlikely to be of physiological significance.

LITERATURE CITED

1. ALBERTY RA 1968 Effect of pH and metal ion concentration on the equilibrium hydrolysis of adenosine triphosphate to adenosine diphosphate. J Biol Chem 243: 1337–1343
2. AMES BN 1966 Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol 8: 115–118
3. BALKE NE, TK HODGES 1975 Plasma membrane adenosine triphosphatase of oat roots. Activation and inhibition by Mg^{2+} and ATP. Plant Physiol 55: 83–86
4. BALKE NE, H SZE, RT LEONARD, TK HODGES 1974 Cation sensitivity of the plasma membrane ATPase of oat roots. *In* J Dainty, U Zimmermann, eds, Membrane Transport in Plants and Plant Organelles. Springer-Verlag, Berlin, pp 301–306
5. BENNETT AB, SD O'NEILL, RM SPANSWICK 1984 H^+-ATPase activity from storage tissue of *Beta vulgaris*. I. Identification and characterization of an anion-sensitive H^+-ATPase. Plant Physiol 74: 538–544
6. BENNETT AB, SPANSWICK RM 1984 H^+-ATPase activity from storage tissue of *Beta vulgaris*. II. H^+/ATP stoichiometry of an anion-sensitive H^+-ATPase. Plant Physiol 74: 545–548
7. BROOKER RJ, CW SLAYMAN 1982 Inhibition of the plasma membrane H^+-ATPase of *Neurospora crassa* by N-ethylmaleimide. J Biol Chem 257: 12051–12055
8. CALDWELL CR, A HAUG 1980 Kinetic characterization of barley root plasma membrane-bound Ca^{2+}- and Mg^{2+}-dependent adenosine triphosphatase activities. Physiol Plant 50: 183–193
9. DUPONT FM, DL GIORGI, RM SPANSWICK 1982 Characterization of a proton-translocating ATPase in microsomal vesicles from corn roots. Plant Physiol 70: 1694–1699
10. ROBERTS JKM 1984 Study of plant metabolism *in vivo* using NMR spectroscopy. Annu Rev Plant Physiol 35: 375–386
11. SEGEL IH 1975 Enzyme Kinetics. John Wiley & Sons, New York, p 206
12. SMITH PT, NA WALKER 1981 Studies on the perfused plasmalemma of *Chara corallina*. I. Current-voltage curves: ATP and potassium dependence. J Membr Biol 60: 223–236
13. SPANSWICK RM 1981 Electrogenic ion pumps. Annu Rev Plant Physiol 32: 267–289
14. STORER AC, A CORNISH-BOWDEN 1976 Concentration of $MgATP^{2-}$ and other ions in solution. Biochem J 159: 1–5
15. THOM M, E KOMOR 1984 Effect of magnesium and ATP on ATPase of sugarcane vacuoles. Planta 161: 361–365
16. WALKER RR, RA LEIGH 1981 Characterisation of salt-stimulated ATPase activity associated with vacuoles isolated from storage roots of red beet (*Beta vulgaris* L.) Planta 153: 140–149