Plant Physiol. (1988) 87, 598–602
0032-0889/88/87/0598/05/$01.00/0

Factors Associated with the Instability of Nitrate-Insensitive Proton Transport by Maize Root Microsomes

Received for publication August 24, 1987 and in revised form March 5, 1988

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ABSTRACT

Proton transport catalyzed by the nitrate-insensitive, vanadate-sensitive H^+-ATPase in microsomes from maize (*Zea mays* L.) roots washed with 0.25 molar KI decreased as a function of time at 0 to 4°C. The rate of proton transport was approximately one-half of that by freshly isolated microsomes after 6 to 18 hours of cold storage. The decrease in proton transport coincided with losses in membrane phosphatidylcholine and was not associated with a change in vanadate-sensitive ATP hydrolysis. A technique based on a protocol developed for the reconstitution of *Neurospora crassa* plasma membrane H^+-ATPase (DS Perlin, K Kasamo, RJ Brooker, CW Slayman 1984 J Biol Chem 259: 7884–7892) was employed to restore proton transport activity to maize microsomes. These results indicated that the decline in proton transport by maize root membranes during cold storage was not due to degradation of the protein moiety of the H^+-ATPase, but was due to the loss of phospholipids.

Membranes from maize roots have been found to contain at least two types of ATP-dependent proton pumping activities mediated by H^+-ATPases (5, 21). One of these H^+-ATPases was associated with the tonoplast and was sensitive to nitrate, while the other was nitrate-insensitive but vanadate-sensitive and believed to be associated with the plasma membrane. Recently, considerable characterization of the tonoplast proton pump has been reported, because tonoplast vesicles with competent transport activities can be isolated by isopycnic density centrifugation (3, 20, 21). However, the vanadate-sensitive proton transport was not recovered after isopycnic centrifugation, although vanadate-sensitive ATP hydrolysis was (21).

The inability to detect vanadate-sensitive proton pumping by membranes after long-term sucrose density gradient centrifugation suggests that some hydrolytic activity was inactivating the plasma membrane proton pump during isolation. A recent report (8) indicated that corn root plasma membranes contain proteolytic activity. Further evidence for the presence of proteases comes from the observation that the electrophoretic pattern of membrane proteins varied with the addition of protease inhibitors and the use of different types of denaturants (7). Therefore, endogenous proteases could be degrading the vanadate-sensitive proton pump during isolation. These proteases would have to hydrolyze the protein moiety of the proton pump in such a way as to inhibit proton transport without affecting vanadate-sensitive ATP hydrolysis.

Membrane fractions from various plants also have been found to contain lipolytic activities (11). If these lipolytic activities caused membranes to be leaky to protons, proton transport could not be detected even though vanadate-sensitive ATP hydrolysis was present. Plasma membranes with proton pumping activity have been isolated from pumpkin and zucchini hypocotyls utilizing phospholipid analogs and nupercaine to minimize the breakdown of lipids (19). To better define a strategy to isolate plasma membranes with proton pumping activity from maize roots, it is necessary to know if the transport activity is inhibited by lipolytic degradation and/or proteolytic digestion during the isolation procedure.

Recently, de Michelis and Spanswick (5) reported two protocols to separate vanadate- and nitrate-sensitive proton pumping activities from maize root microsomes. One method utilized a 2 h sucrose density gradient centrifugation containing various protectants. However, the purity of the putative plasma membrane fraction was not established. The second protocol significantly reduced the activity of the nitrate-sensitive proton pumping by washing microsomes with 0.25 M KI. To facilitate the purification of plasma membranes with proton pumping activity from 0.25 M KI-washed microsomes, the stability of proton transport at 0 to 4°C was investigated. The results of this report suggest that the loss of proton pumping with incubation was due to lipolytic degradation, and this proton pumping activity readily could be restored by a reconstitution method.

MATERIALS AND METHODS

Plant Material. *Zea mays* L. seeds (cv WF9 × Mo17, from Crow Hybrid Seed Co.[1]) were germinated on filter paper moistened with 0.1 mM $CaCl_2$ for 3 to 4 d at 28 to 30°C and were harvested as described previously (13). Seeds were soaked in 10% Clorox for 10 min with agitation by magnetic stirrer, and then rinsed for 30 min in cool water prior to planting to eliminate fungal contamination.

Preparation of Microsomal Membranes. Microsomal membranes were isolated by modifying the protocol of de Michelis and Spanswick (5). Roots were thoroughly chopped in ice-cold homogenization buffer (0.25 M sucrose, 10% [w/v] glycerol, 2 mM EGTA, 2 mM $MgSO_4$, 2 mM ATP, 1 mM PMSF[2], 0.5% BSA, 5 mM DTT, and 25 mM BTP titrated to pH 7.8 with Mes) using 3 to 4 ml of buffer per g fresh weight of roots and then homogenized by mortar and pestle for 4 to 5 min at 0 to 4°C. After filtering through cheesecloth, the brei was centrifuged at 12,000*g* for 12 min and the resulting supernatant was centrifuged again for 35 min at 90,000*g*. For KI-washed microsomes, the 90,000*g*

[1] Reference to brand or firm does not constitute endorsement by the United States Department of Agriculture over others of a similar nature not mentioned.

[2] Abbreviations: PMSF, phenylmethylsulfonyl fluoride; AO, acridine orange; PC, phosphatidylcholine; DOC, Na salt of deoxycholic acid; BTP, bis-tris-propane or 1,3-bis[tris(hydroxymethyl)-methylamino] propane.

pellet was resuspended in homogenization buffer containing 0.25 M KI using 1 ml of buffer per g of roots. After holding on ice for 10 min, the 90,000*g* centrifugation was repeated. Microsomes and KI-washed microsomes were dispersed in resuspension buffer containing 0.25 M sucrose, 10% (w/v) glycerol, 2 mM DTT, and 2 mM BTP titrated to pH 7.2 with Mes using 1 ml of buffer per 5 g of roots. The protein concentration of such suspensions varied between 2 and 4 mg/ml. Membrane aliquots were routinely stored up to 4 weeks at −20°C without loss in ATP-hydrolyzing activity.

ATP Hydrolysis Assay. ATP hydrolysis was assayed by a modification of the protocol of Addision and Scarborough (1). Ten μl of membrane suspensions were added to 85 μl of reaction mixture for 3 min at 18 to 22°C. The reaction was initiated by the addition of 5 μl of 0.2 M ATP titrated to pH 6.45 with BTP. The reaction medium had final concentrations of 40 mM Mes-Tris (pH 6.45), 10 mM $MgSO_4$, 1 mM EGTA, 5 mM NaN_3, and either 50 mM KCl or 50 mM KNO_3 with and without 0.2 mM sodium orthovanadate. The reaction was terminated by the addition of 100 μl of ice-cold 5% TCA, and the tubes were held on ice. The amount of inorganic phosphate released was determined by the malachite green assay as described previously (21). The activities of the tonoplast and plasma membrane ATPases were determined by the nitrate- and vanadate-sensitive components of ATP hydrolysis, respectively.

ATP-Dependent Proton Uptake Assay. Proton transport was followed by changes in the absorbance of AO at 492 nm as described by de Michelis *et al.* (4). Typically, 200 μl of vesicles were diluted with 2 ml of 17.5 mM Mes-Tris (pH 6.45), 2.5 mM $MgSO_4$, 1 mM EGTA, 7.5 μM AO, and either 50 mM KCl or 50 mM KNO_3. After equilibrating at room temperature for 5 min, the reaction was initiated by the addition of 20 μl of 0.2 M ATP titrated to pH 6.45 with BTP.

Autolysis of Membrane Lipids. Lipolytic acyl hydrolyase activity was followed by the procedure of Moreau and Isett (12), which quantifies the loss of lipid components as a function of time at 0 to 4°C. Lipids in 0.2 ml aliquots were extracted with 7 ml of 3:2 (v/v) hexane to isopropanol and the organic phase was washed with 5 ml of 6.7% Na_2SO_4. The organic phase was dried with gentle heat under a stream of N_2, suspended in 1:1 (v/v) chloroform to methanol, and spotted onto 250 μm silica gel G TLC plates. TLC plates were developed in 85:15:10:3.5 (v/v/v/v) chloroform, methanol, acetic acid, and water, and the lipid components were visualized with I_2. Spots which chromatographed like the corresponding standards were scraped from the plate and subjected to total phosphate analysis by the method of Dittmer and Wells (6).

Reconstitution Protocol. A procedure to reconstitute vanadate-sensitive proton pumping was adapted from the protocol developed by Perlin *et al.* (17) for the *Neurospora crassa* plasma membrane ATPase. Asolectin was partially purified and stored in chloroform at −20°C as described by Kawaga and Racker (9). The day prior to reconstitution, the chloroform was removed from the asolectin under a gentle stream of N_2 and the dried lipid film was stored *in vacuo* overnight. A sufficient volume of reconstitution buffer (10 mM Mes-Tris, [pH 6.45], 50 mM K-acetate, and 5 mM NaN_3) was added to the dried lipid to yield 40 mg of asolectin per ml, and the suspension was sonicated to clarity in a bath-type sonicator. Typically, 0.8 mg of microsomal protein were diluted with resuspension buffer to 0.7 ml and 12 mg of asolectin in 0.3 ml of reconstitution buffer were added. The membranes and asolectin were then dispersed by the addition of 10% (w/v) DOC to a final concentration of 0.6%. The detergent-protein-lipid solution was applied to the top of a Sephadex G-150 column (1.5 × 20 cm) equilibrated with reconstitution buffer. The column was eluted with reconstitution buffer at a flow rate of 1 ml/min at room temperature. The cloudy void volume fractions (typical ml 9–12) were pooled and stored on ice until assayed.

Electrophoresis. SDS-PAGE was performed by a modification of the Laemmli protocol as described by Addison and Scarborough (1). Membrane samples were disaggregated by diluting with an equal volume of SDS sample buffer (50 mM Tris-PO_4 [pH 6.8], 10% (w/v) glycerol, 1% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 1 mM EDTA, and 10 μg/ml chymostatin) and immediately placed into a boiling water bath for 3 min.

Protein Assay. Protein content was determined after deoxycholate-TCA precipitation by the Lowry method (2).

RESULTS AND DISCUSSION

Preliminary Observations with KI-Washed Microsomes. Proton transport activity was found to decline markedly with storage at 0 to 4°C such that less than 10% of the initial activity routinely remained after 24 h. Proteolytic degradation of exogenously added proteins and of membrane polypeptides has been reported previously with maize root plasma membranes (7, 8). However, there was no evidence of dramatic changes in the polypeptide composition of KI-washed microsomes over a 16 h period as determined by SDS-PAGE (Fig. 1). In this experiment, freshly isolated microsomes had a proton transport activity of 0.034 ΔA/min/mg protein. The lack of effect of incubation on the polypeptide composition was interpreted as evidence against proteolytic inactivation. This conclusion was dependent on the

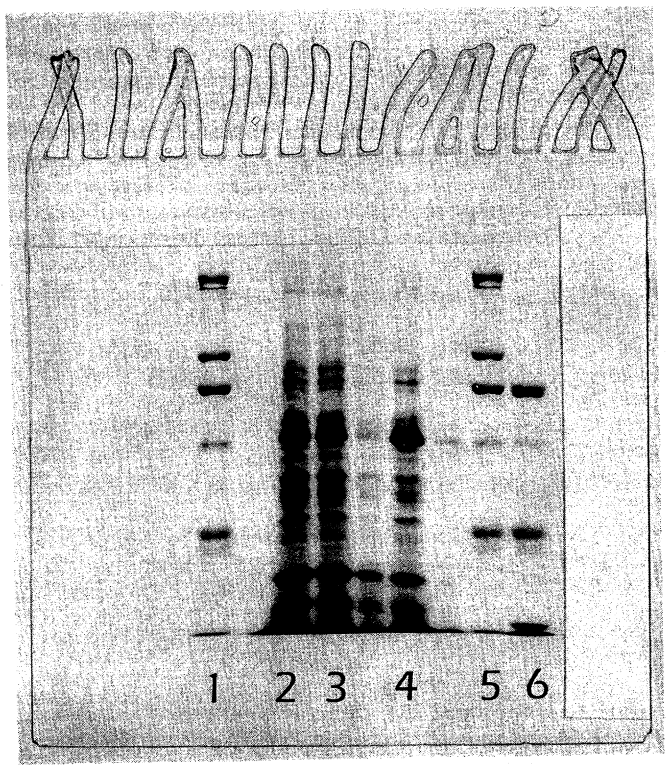

FIG. 1. SDS-PAGE analysis of proteins from KI-washed microsomes. Aliquots of microsomes containing 100 μg of protein that either were incubated 0 or 16 h at 0 to 4°C or were disaggregated and subjected to electrophoresis as described in "Materials and Methods." Lanes 2 and 3 depict membrane proteins after 0 and 16 h, respectively. Lanes 1 and 5 depict standards of 200, 116, 97.4, 66.2, and 42.7 kD and lane 6 standards of 97.4, 66.2, 42.7, 31.0, 21.5, and 14.4 kD. Forty μg of protein from vesicles reconstituted from KI-washed microsomes appear in lane 4. Staining material between numbered lanes represents contamination to empty lanes.

assumption that the degree of proteolytic cleavage during disaggregation and electrophoresis was not of the magnitude to obscure the effects of incubation. Gallagher and Leonard (7) recently have suggested that SDS-PAGE may not be the best method for electrophoretic analysis of maize membrane proteins because of the extent of proteolytic degradation during sample preparation. From the data of Gallagher and Leonard (7), our use of the protease inhibitor chymostatin and of disaggregation in a boiling bath should have minimized proteolytic degradation during sample preparation. Differences between this report and that of Gallagher and Leonard (7) may reflect subtle differences between membranes from roots of 3-d-old seedlings used in this study *versus* 14- to 21-d-old seedlings. The notion that proteolytic degradation was not involved in the loss of proton transport activity was further supported by the lack of an effect of added chymostatin, PMSF, and BSA on the stability of this activity.

The permeability of microsomes to protons was found to increase with storage as assayed by 'pH jump' experiments (Fig. 2). When freshly isolated microsomes were suspended in the proton pumping assay media and the pH of the external solution was increased to pH 7.5 by the addition of base, the collapse of the pH gradient as followed by changes in AO absorbance occurred over a few minutes. Similar rates of proton diffusion have been published previously for membranes from maize roots (16). When microsomes were assayed after 16 h at 0 to 4°C, there was no time-dependent change in absorbance after the addition of base, indicating the membranes could not support a pH gradient.

When classes of lipids were separated by TLC, it was apparent that most of the lipid constituents declined during cold storage (Fig. 3). There was no increase in the levels of lyso-PC and phosphatic acid corresponding to the decline in PC, thus eliminating the possibility that either phospholipase A_2 or D was the sole means of lipid degradation. The results of these experiments

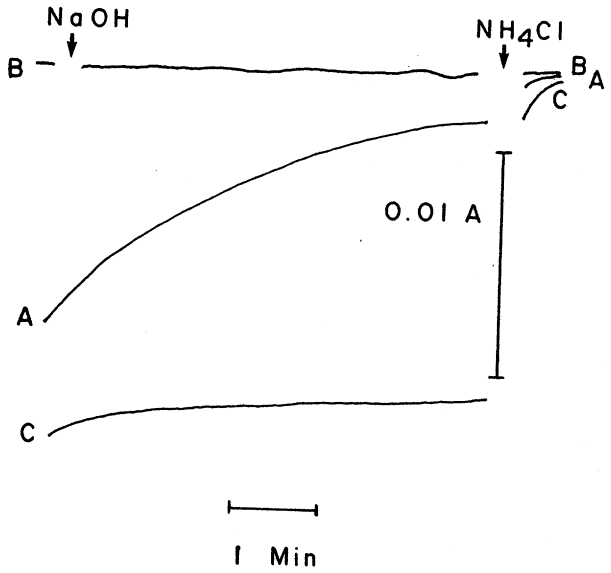

FIG. 2. Changes in AO absorbance in response to the application of a pH gradient. Four hundred μg of microsomal protein in 0.2 ml were diluted with 2 ml of proton transport assay media. After equilibrating at room temperature for 5 min, absorbance at 492 nm was monitored and the pH of the buffer was raised to pH 7.5 by the addition of 20 μl of 2 M NaOH at the indicated time. In trace A, freshly isolated microsomes were assayed, whereas in trace B microsomes that had been incubated 16 h at 0 to 4°C were used. In trace C, a similar study was conducted using 50 μg of reconstituted microsomal protein except that the pH gradient remaining after 5 min was dissipated by the addition of 20 μl of 1 M NH_4Cl at the indicated time.

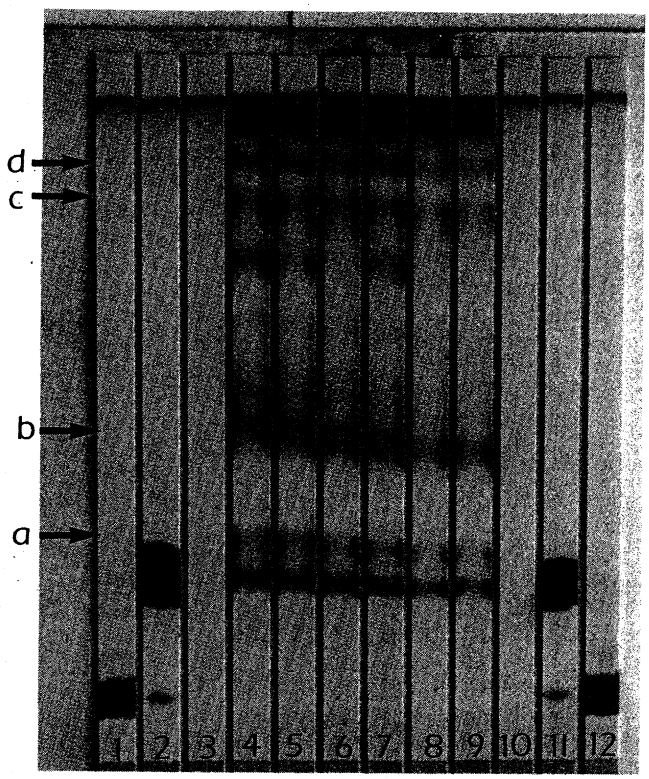

FIG. 3. Changes in lipid classes as a function of time at 0 to 4°C. Lipids in 1 ml aliquots of KI-washed microsomes were extracted for phospholipid analysis at 8 h intervals as described in "Materials and Methods." After developing the TLC plate, lipids were visualized with I_2 vapor. Lanes 1 and 2 contained aliquots of lyso-PC and PC, respectively. Lanes 4 through 9 contain the lipids present in microsomes after 0, 8, 16, 24, 32, and 40 h of cold storage. The two standards are repeated in lanes 11 and 12. Locations marked at arrows labeled a, b, c, and d indicate the relative migration of preparations of digalactose diacylglycerol, phosphatidylethanolamine, monogalactose diacylglycerol, and phosphatic acid, respectively. At 0 h, there were 380 nmol PC/mg protein which declined to 79, 64, 51, 40 and 32% of initial value after 8, 16, 24, 36, and 40 h, respectively.

suggested that the loss in proton pumping was associated with a loss of lipid constituents and enhanced permeability to protons.

Development of the Reconstitution Protocol. If proton transport activity was lost because the membrane lipids were degraded, then a reconstitution protocol to replace degraded lipids should restore proton transport activity. Procedures utilizing DOC were investigated because the plasma membrane ATPase from roots is not readily denatured by this detergent (14). Perlin *et al.* (17) developed a protocol to reconstitute the H^+-ATPase from plasma membranes of *N. crassa* in which the proteins were not solubilized prior to reconstitution. In this protocol, plasma membrane vesicles in the presence of added lipid were clarified by adding DOC to a final concentration of 0.6%. Proteoliposomes were formed by removing the detergent by gel filtration. When KI-washed microsomes from maize roots were treated in a similar fashion using 0.8 mg of protein and 12 mg of asolectin, the resulting proteoliposomes exhibited ATP-dependent, nitrate-insensitive, vanadate-sensitive proton transport (Fig. 4). These proteoliposomes were found to have a low permeability to protons as indicated by the stability of an imposed pH gradient and its collapse by NH_4Cl (Fig. 2). The effect of protein and asolectin concentrations on the efficiency of the reconstitution protocol was determined. The activity of proton transport increased linearly with the amount of protein added up to at least 1.2 mg,

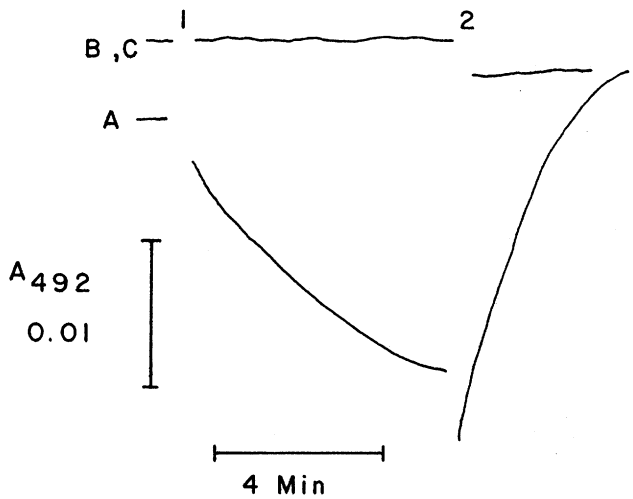

FIG. 4. Proton transport activity of reconstituted vesicles as followed by changes in AO absorbance. Eight hundred μg of KI-washed microsomal protein were combined with 12 mg of asolectin, dispersed by the addition of DOC to 0.6% and applied to G-150 column. The void volume fractions were pooled, and 200 μl were assayed for proton transport activity in the presence of 50 mM KNO_3. In trace A, at points 1 and 2, respectively, were added 20 μl of 0.2 M ATP-BTP (pH 6.45) and 20 μl of 20 μg/ml nigericin. In trace B, the assay media contained 0.2 mM sodium vanadate prior to the addition of ATP. In trace C, no ATP was added.

and a minimum of 10 mg of asolectin per mg of protein was necessary for optimum rates of proton transport by the proteoliposomes (data not shown).

To determine if the reconstituted ATPase activity was associated with lipid vesicles, proteoliposomes and liposomes were subjected to isopycnic glycerol gradient centrifugation (data not shown). Proteoliposomes equilibrated as a sharp peak on a gradient of 10 to 30% (w/v) glycerol with an average density of 1.064 g/cm^3. Approximately three-fourths of the added phospholipids were recovered. There was a close correspondence between the distribution of phospholipids and vanadate-sensitive ATPase activity among the fractions from the glycerol gradient. Virtually all of the ATPase activity was recovered. The microsomes prior to reconstitution contained 112 nmol Pi/min of ATPase activity, of which 102 were recovered in the fractions with phospholipids. These results indicated that a majority of the ATPase activity was recovered and membrane bound. Most of the predominant polypeptides in KI-washed microsomes were recovered upon reconstitution (Fig. 1, lane 4).

During reconstitution, a substantial amount of the nonspecific phosphatases present in KI-washed microsomes appeared to be inactivated. The level of ATP hydrolysis in the presence of nitrate and vanadate was reduced from 26 to 4 nmol Pi/min by reconstitution. The activity of the nitrate-sensitive H^+-ATPase also was reduced after reconstitution. Although this effect was observed with KI-washed microsomes, it was more noticeable when crude microsomes were reconstituted. Crude microsomes exhibited both nitrate- and vanadate-sensitive proton transport. Nitrate-sensitive proton transport was substantially greater, 0.132 *versus* 0.024 ΔA/min/mg protein for the nitrate-insensitive activity. However, no nitrate-sensitive proton pumping could be detected after reconstitution. The activity of the nitrate-resistant, vanadate-sensitive activity was approximately the same before and after reconstitution, 0.024 and 0.025 ΔA/min/mg protein, respectively. Preliminary experiments indicated that the nitrate-sensitive proton pump was irreversibly inhibited by the level of DOC used in the reconstitution procedure (data not shown). This inactivation by DOC may stem from the fact that this H^+-ATPase is composed of multiple subunits (18) which may not reassociate after being exposed to DOC.

Changes in Enzyme Activity with Incubation at 0 to 4°C. The reconstitution protocol described in this report can nearly quantitatively incorporate functional vanadate-sensitive H^+-ATPase into asolectin vesicles. Therefore, proton transport activity by reconstituted vesicles should be a reliable measure of the potential proton transporting activity of the microsomal preparation. To further establish that the loss in proton transport activity during incubation at 0 to 4°C was not due to an irreversible damage of the H^+-ATPase, KI-washed microsomes were reconstituted after 0, 8, 16, and 24 h of incubation and assayed for proton transport, ATP hydrolysis, and PC content (Fig. 5). Proton transport by native vesicles declined to 20% of initial value, from 0.035 to 0.008 ΔA/min/mg protein over 24 h. The loss of membrane lipids was quantified by analyzing the amount of PC remaining in the microsomes after 8 to 24 h of incubation. Levels of PC in the membranes decreased to 60% of initial value from 370 to 230 nmol PC/mg protein following a time course similar to the loss of proton pumping. Vanadate-sensitive ATP hydrolysis was relatively constant, averaging 56 nmol Pi/min/mg protein. Proton transport by reconstituted proteoliposomes of freshly isolated KI-washed microsomes was 0.042 ΔA/min/mg protein, slightly greater than that observed with the vesicles prior to reconstitution. Proton transport by vesicles reconstituted after 8, 16, and 24 h of storage at 0 to 4°C was within 5% of initial values averaging 0.044, 0.042, and 0.041 ΔA/min/mg protein at the respective time intervals. Thus, the reconstitution protocol restored proton pumping activity, and the level of this activity was independent of time at 0 to 4°C.

The loss in proton transport activity by KI-washed microsomes

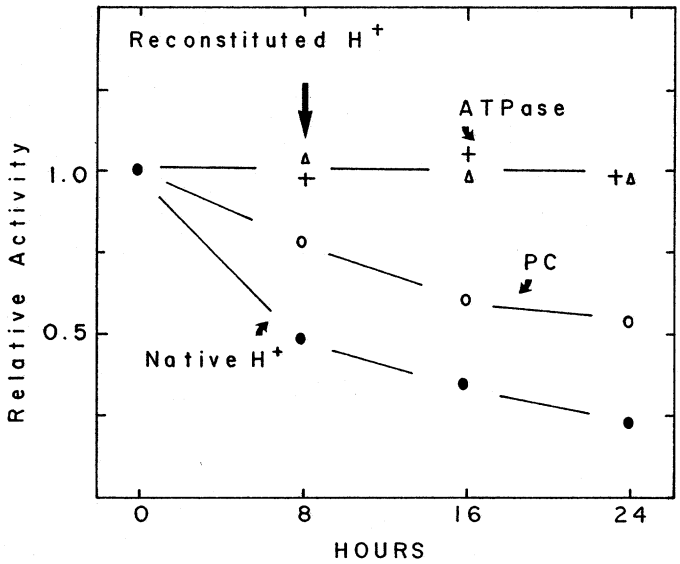

FIG. 5. Changes in proton transport by native and reconstituted KI-washed microsomes, PC content, and vanadate-sensitive ATP hydrolysis over 24 h incubation at 0 to 4°C. On 8 h intervals, aliquots containing 0.6 mg of microsomal protein were reconstituted with 12 mg of asolectin as described in "Materials and Methods" and assayed for protein pumping in the presence of 50 mM KNO_3. Native vesicles were also assayed for proton transport in the presence of 50 mM KNO_3. At 0 h values, initial rates of proton transport were 0.035 and 0.042 ΔA/min/mg protein for native and reconstituted vesicles, respectively; the PC content was 370 nmol/mg protein; and the vanadate-sensitive ATPase activity was 56 nmol/min/mg protein. Values for proton transport by native and reconstituted vesicles are represented by (●) and (Δ), respectively; for vanadate-sensitive ATP hydrolysis by (+); and for PC content by (O).

appeared to be due to a loss in membrane integrity caused by the degradation of endogenous lipids (Fig. 3). Preliminary experiments characterizing the collapse of an imposed pH gradient indicated that the proton permeability of microsomes increased with cold storage (Fig. 2). Therefore, the loss of proton transport may have resulted from the inability to establish a proton gradient due to enhanced proton leak. The possibility that the loss of proton transport was due to the degradation of specific lipids required for the activation of the ATPase or some other proteins important for maintaining membrane integrity cannot be ruled out. Recently, Xie *et al.* (22) reported that the phospholipid requirements for proton transport by the clathrin-coated vesicles were different from those of ATP hydrolysis. However, such an explanation does not seem plausible for the nitrate-insensitive proton pump from maize roots from preliminary experiments which indicate that proton transport activity of microsomes and DOC-extracted microsomes can be reconstituted using a variety of highly purified, synthetic phospholipids (D Brauer, unpublished data). The adapted reconstitution protocol restored proton transport activity to vesicles that had lost most of their initial activity (Fig. 5).

These results strongly support the contention that the loss of proton transport activity was primarily associated with the degradation of membrane phospholipids. The time course for losses in proton transport activity and PC were similar but of different magnitudes, *i.e.*, 80% loss in transport activity over 24 h *versus* 40% loss in PC content (Fig. 5). In light of these results, our attempts to isolate plasma membrane vesicles with stable transport activities will focus on the reduction of the autolytic lipase activity. Autolytic acyl hydrolase activity from other plant membrane tissues has been shown to be highly regulated including modulation by calmodulin (10, 15). Disruption of the activation of phospholipid degradation by the inclusion of calmodulin antagonists may provide one means for purifying plasma membrane vesicles with competent proton transport by isopycnic density centrifugation.

Acknowledgments—The authors thank Dr. Bob Moreau of the Eastern Regional Research Center for his excellent assistance in the area of autolytic phospholipid degradation and our student trainee, Chris Thomas.

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