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Characterization of Native and Reconstituted Plasma Membrane H^+-ATPase from the Plasma Membrane of *Beta vulgaris*

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Summary. Characteristics of the native and reconstituted H^+-ATPase from the plasma membrane of red beet (*Beta vulgaris* L.) were examined. The partially purified, reconstituted H^+-ATPase retained characteristics similar to those of the native plasma membrane H^+-ATPase following reconstitution into proteoliposomes. ATPase activity and H^+ transport of both enzymes were inhibited by vanadate, DCCD, DES and mersalyl. Slight inhibition of ATPase activity associated with native plasma membranes by oligomycin, azide, molybdate or NO_3^- was eliminated during solubilization and reconstitution, indicating the loss of contaminating ATPase activities. Both native and reconstituted ATPase activities and H^+ transport showed a pH optimum of 6.5, required a divalent cation ($Co^{2+} > Mg^{2+} > Mn^{2+} > Zn^{2+} > Ca^{2+}$), and preferred ATP as substrate. The Mg:ATP kinetics of the two ATPase activities were similar, showing simple Michaelis-Menten kinetics. Saturation occurred between 3 and 5 mM Mg:ATP, with a K_m of 0.33 and 0.46 mM Mg:ATP for the native and reconstituted enzymes, respectively. The temperature optimum for the ATPase was shifted from 45 to 35°C following reconstitution. Both native and reconstituted H^+-ATPases were stimulated by monovalent ions. Native plasma membrane H^+-ATPase showed an order of cation preference of $K^+ > NH_4^+ > Rb^+ > Na^+ > Cs^+ > Li^+ >$ choline$^+$. This basic order was unchanged following reconstitution, with K^+, NH_4^+, Rb^+ and Cs^+ being the preferred cations. Both enzymes were also stimulated by anions although to a lesser degree. The order of anion preference differed between the two enzymes. Salt stimulation of ATPase activity was enhanced greatly following reconstitution. Stimulation by KCl was 26% for native ATPase activity, increasing to 228% for reconstituted ATPase activity. In terms of H^+ transport, both enzymes required a cation such as K^+ for maximal transport activity, but were stimulated preferentially by Cl^- even in the presence of valinomycin. This suggests that the stimulatory effect of anions on enzyme activity is not simply as a permeant anion, dissipating a positive interior membrane potential, but may involve a direct anion activation of the plasma membrane H^+-ATPase.

Key Words H^+-ATPase · plant plasma membrane · reconstitution · vanadate · red beet

Introduction

In the accompanying paper, we have demonstrated the activity of a reconstituted H^+-ATPase derived from plasma membrane of red beet (O'Neill & Spanswick, 1984). This reconstituted H^+-ATPase was enriched three- to fourfold in specific activity relative to native plasma membrane and was relatively free of tonoplast and mitochondrial H^+-ATPases that contaminate the plasma membrane fraction. Both the reconstituted H^+-ATPase and the native plasma membrane H^+-ATPase were strongly inhibited by vanadate. The ATPase activities of these two fractions was half-maximally inhibited by less than 10 μM vanadate. For the reconstituted enzyme, a K_i of 2.6 μM vanadate (Na_3VO_4) was determined for ATPase activity. The reconstituted H^+ transport was equally sensitive to inhibition by vanadate, being inhibited half-maximally between 1 and 5 μM (O'Neill & Spanswick, 1984).

The criterion of vanadate inhibition has been relied on in our work on H^+-translocating ATPases from red beets as a means of identifying that activity derived from the plasma membrane rather than from the tonoplast or mitochondria. It has permitted us to group the native and reconstituted H^+-ATPase of the red beet plasma membrane in with the other major ion translocating ATPases, all of which are inhibited by less than 20 μM vanadate (Macara, 1980). These enzymes form a phosphorylated intermediate during the course of their reaction cycle with which vanadate interferes (Cantley, Cantley & Josephson, 1978; Dame & Scarborough, 1980; Briskin & Poole, 1983*b*).

Other criteria, though, may also be important in identifying the native and reconstituted H^+-ATPase as the primary ion translocating ATPase of the plant plasma membrane. These include kinetic properties of the enzyme (pH and temperature optima, substrate specificity, Mg:ATP kinetics, and salt requirements) as well as inhibitor sensitivity. Biochemical characterization of the reconstituted H^+-ATPase is now possible given that this *in vitro* system is relatively free of other contaminating H^+-

ATPases and nonspecific phosphatases. The H^+ pump is also active in the reconstituted system, permitting a detailed characterization of transport properties of the isolated plasma membrane H^+-ATPase. In this report, we have characterized the biochemical properties of the reconstituted H^+-ATPase and have compared these throughout with those of the native plasma membrane H^+-ATPase in terms of both ATPase activity and H^+ transport.

Materials and Methods

Membrane Preparation

Plasma membranes were prepared from red beet (*Beta vulgaris* L.) as described in the accompanying paper (O'Neill & Spanswick, 1984). In brief, the plasma membrane fraction was collected from discontinuous sucrose gradients at the 34/38% (wt/wt) interface following centrifugation for 2 hr at 80,000 × *g* in a Beckman SW 27 rotor. The 34/38% (wt/wt) interface was collected, diluted, repelleted, and resuspended in solubilization buffer containing 45% glycerol prior to solubilization and reconstitution.

Solubilization and Reconstitution

Plasma membranes were resuspended to a final protein concentration of 1.5 mg/ml in solubilization buffer. Deoxycholate was added from a 10% stock solution to a final concentration of 0.3% resulting in an overall detergent/protein ratio (wt/wt) of 2. Following centrifugation, a solubilized enzyme extract was obtained and used for reconstitution. Reconstitution was accomplished by removing the detergent from the solubilized enzyme-phospholipid sample by gel filtration as described previously (O'Neill & Spanswick, 1984). The reconstituted proteoliposomes were resuspended in 2.5 mM Tris/Mes (pH 6.5) and 1 mM DTT and used directly for transport and ATPase assays. When freezing and storage overnight of the reconstituted enzyme was necessary, the pelleted proteoliposomes were resuspended in 2.5 mM Tris/Mes (pH 6.5), 1 mM DTT and 10% glycerol or 250 mM sucrose. Both plasma membranes and reconstituted vesicles were frozen in liquid N_2 and stored at −70°C until use. Enzyme activity was maintained when samples were prepared and handled in this manner.

Transport Assays

H^+ transport was measured continuously by monitoring the quenching of fluorescence of the permeant amine dyes, quinacrine or acridine orange as described previously (O'Neill & Spanswick, 1984). The basic reaction mix contained 10 mM Tris/Mes (pH 6.5), appropriate salts and/or inhibitors, and either 10 μM quinacrine or 5 μM acridine orange. For assaying the effect of pH on H^+ transport, the pH of the reaction mix was varied by titrating 10 mM Tris and Mes buffers to achieve a graded series between pH 5.0 and 8.5. Equilibration between the external medium and the internal medium of the vesicles was achieved before initiating the transport assay with ATP : Mg. Approximately 70 or 35 μg of membrane protein were added per transport assay for native plasma membrane or reconstituted vesicles, respectively. The initial interaction between the acridine dye and ATP, amounting to about 10 to 15% of the total fluorescence, has been corrected for in the quench curves.

Enzyme Assays

ATPase activity was determined by measuring the release of inorganic phosphate from ATP (Tris salt) according to the method of Ames (1966). ATPase activity was assayed for 30 min at 28°C except for the determination of temperature optima, in which case activity was assayed for 10 min at the appropriate temperature. The basic reaction mix contained 5 mM ATP/Tris (pH 6.5), 5 mM $MgSO_4$, 30 mM Tris/Mes (pH 6.5), appropriate salts, plus or minus inhibitors and/or ionophores in a final volume of 0.5 ml. The pH of the reaction mix was adjusted to 6.5 at the assay temperature. For determining the effect of pH on ATPase activity, the pH of the ATP/Tris, the composition of the 30 mM Tris/Mes buffer, and the final reaction mix was adjusted to the desired pH. In many of the ATPase assays, 50 to 100 mM KNO_3 was included to inhibit any contaminating tonoplast ATPase activity (O'Neill, Bennett & Spanswick, 1983). Appropriate controls and enzyme blanks were included in all inhibitor, ionophore and salt effect assays. For the phosphohydrolase assays, substrates were supplied as 5 mM Na^+ salts.

Protein Determination

Proteins were determined by the method of Schaffner and Weissmann (1973).

Source of Chemicals

ATP was obtained from Boehringer Mannheim as the disodium salt and converted to the Tris salt by filtration through Dowex 50W. Sodium orthovanadate (Na_3VO_4) was obtained from Fisher Scientific Co. and vanadium oxide (V_2O_5), gold label, from Aldrich Chemical Co. The concentrations of the vanadate stock solutions were verified spectrophotometrically using the extinction coefficient of Cantley et al. (1977). DCCD, FCCP, gramicidin, nigericin and valinomycin were obtained from Calbiochem-Behring. All other chemicals were obtained from Sigma.

Results and Discussion

Inhibitor Sensitivity

The effect of inhibitors on ATPase activity associated with the native plasma membrane and reconstituted ATPase is summarized in Table 1. Both enzymes were inhibited to a similar extent by DCCD (50 μM), DES (50 μM), and EDAC (1 mM). Small amounts of inhibition of plasma membrane ATPase activity by oligomycin (5 μg/ml), azide (1 mM), molybdate (0.1 mM), and NO_3^- (50 mM) were eliminated during the solubilization/reconstitution procedure indicating that the reconstituted system was rela-

Table 1. Effect of inhibitors on ATPase activity associated with red beet plasma membrane and reconstituted ATPase

Inhibitor	Assay conc.	ATPase activity[a]			
		Plasma membrane		Reconstituted ATPase	
		Specific activity (μmol P_i mg^{-1} hr^{-1})	% of control	Specific activity (μmol P_i mg^{-1} hr^{-1})	% of control
Control	—	40.3	—	80.0	—
+Gramicidin	2 μM	48.4	100	85.2	100
+DCCD	50 μM	10.8	22	25.3	30
+DES	50 μM	24.5	51	42.4	50
+Oligomycin	5 μg/ml	44.5	92	89.3	105
+Fusicoccin	5 μM	45.5	94	87.9	103
+EDAC	1 mM	43.3	89	74.3	87
+Mersalyl	50 μM	46.1	95	69.9	82
+NaN_3	1 mM	45.2	93	90.0	106
+Molybdate	0.1 mM	46.0	95	84.7	99
+Vanadate	50 μM	10.5	22	11.0	13
+KNO_3	50 mM	37.9	78	92.8	109

[a] ATPase activity was determined in the presence of 5 mM ATP, 5 mM $MgSO_4$, 30 mM Tris/Mes, 50 mM KCl, ±2 μM gramicidin at pH 6.5 and 28°C.

tively free of contaminating enzyme activities such as mitochondrial ATPase, tonoplast ATPase and nonspecific phosphatases. The reconstituted enzyme showed an enhanced sensitivity to inhibition by vanadate and the mercurial mersalyl. It should be noted, however, that the presence of 50 μM DTT during the ATPase assay may have affected our results with mersalyl. This is not expected though since this concentration of DTT is much lower than that typically required to reduce disulfides. The data overall suggest that solubilization and reconstitution of the plasma membrane H^+-ATPase results in a partial purification of the enzyme leading to elimination of inhibition by various inhibitors of other cellular ATPases. The most potent and selective inhibitor of the red beet plasma membrane H^+-ATPase was vanadate, which inhibited ATPase activity of the enzyme approximately 90% at a concentration of 50 μM. The effect of vanadate on both ATPase activity and H^+ transport of the native plasma membrane and reconstituted H^+-ATPase was presented in detail in the accompanying paper (O'Neill & Spanswick, 1984).

The effects of various inhibitors on H^+ transport of native plasma membrane vesicles were presented in a related paper (Bennett, O'Neill & Spanswick, 1984). In brief, H^+ transport associated with the native plasma membrane vesicles was inhibited by vanadate (100 μM), but not by oligomycin (5 μg/ml) or NO_3^- (100 mM). H^+ transport was also sensitive to two other inhibitors, DCCD and DES, and these also inhibited the plasma membrane ATPase activity (*unpublished results*). Here, we examined the effect of various inhibitors on H^+ transport associated with the reconstituted proteoliposomes (Fig. 1). Reconstituted vesicles were preincubated in a reaction mix containing 100 mM KNO_3 with or without the inhibitor. Transport was insensitive to NO_3^- (100 mM) indicating that the H^+-ATPase was not of tonoplast origin. Further characterization of the inhibitor sensitivity of the reconstituted H^+-ATPase was carried out in the presence of NO_3^- as a safeguard against measuring any contaminating tonoplast H^+ transport activity. Fluorescence quenching was nearly abolished by vanadate (100 μM). DCCD (50 μM) and DES (100 μM) were also effective inhibitors. However, oligomycin (5 μg/ml), an inhibitor of submitochondrial particle H^+ transport, (Thayer & Hinkle, 1973), had no effect. Two other compounds that supposedly stimulate the plasma membrane proton pump *in vivo*, fusicoccin (5 μM) and indoleactic acid (10 μM), were without effect on reconstituted H^+ transport. Various ionophores abolished fluorescence quenching, including the combination of FCCP and valinomycin as shown. These inhibitor characteristics clearly distinguish H^+ transport activity of this reconstituted H^+-ATPase from that of the tonoplast H^+-ATPase derived from the same red beet tissue (Ben-

nett et al., 1984) and from that of corn root anion-sensitive H^+-ATPase (Bennett & Spanswick, 1983*b*).

Optimum pH and Temperature

A criterion that has been important in identifying the plasma membrane ATPase in plants is the pH optimum of 6.5. Figure 2 shows the effect of pH on both ATPase activity and H^+ transport. Native plasma membranes had an optimum pH of 6.5 for both ATPase and H^+ transport. Following reconstitution, the specific activity of the enzyme increased, yet the basic pH profile for ATPase activity was the same. H^+ transport also retained a pH optimum of 6.5 although the pH profile was much sharper. The apparent enhancement of H^+ transport at the optimal pH (6.5) may have resulted from the use of initial rates of fluorescence quench in assessing H^+ transport. The initial rate of quench is proportional to the initial rate of H^+ influx and to the internal buffer strength (Bennett & Spanswick, 1983*a*). Since the buffer strength is pH dependent, initial rates of fluorescence quench may not be strictly comparable at different initial pH values. This may have contributed to the sharper pH profile for H^+ transport relative to ATPase activity.

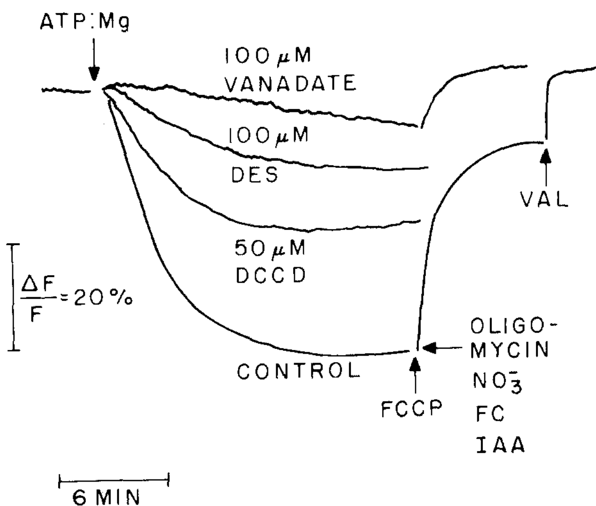

Fig. 1. Effect of inhibitors on H^+ transport associated with red beet reconstituted H^+-ATPase. Fluorescence quench assays were carried out in the presence of vesicles, 5 μM acridine orange, 10 mM Tris/Mes (pH 6.5), 100 mM KNO_3 and inhibitor to the final concentration listed. The concentration of oligomycin was 5 μg/ml. Fusicoccin (FC) and indoleacetic acid (IAA) were also tested at 5 μM and 10 μM final concentrations, respectively. ATP : Mg (5 mM), FCCP (0.5 μM), and valinomycin (0.5 μM) were added where indicated. Approximately 30 μg membrane protein were used per quench assay

We also compared the temperature sensitivity of the native plasma membrane and reconstituted enzymes (Fig. 3). Plasma membrane ATPase activity increased steadily between 20 and 45°C with a Q_{10} of 2.6 between 20 and 30°C. Activity reached a maximum at 45°C, declining slightly at 50°C. The reconstituted ATPase had an altered temperature profile with a maximum for ATPase activity at 35°C. The specific activity at this temperature exceeded 250 μmol P_i mg^{-1} h^{-1}. Activity declined sharply between 35 and 50°C. A Q_{10} of 4.6 was calculated for the change in enzyme activity between 20 and 30°C, a value which was substantially greater than that observed over the same temperature span for the native plasma membrane enzyme. Over this temperature range, the activities of both enzymes increased linearly. The change in the temperature profile for ATPase activity following reconstitution indicates that the enzymes temperature coefficient has been altered by solubilization and reconstitution. Its decline above 35°C suggests a loss of stability at higher temperatures. The reason for these changes is not clear. However, it is most likely re-

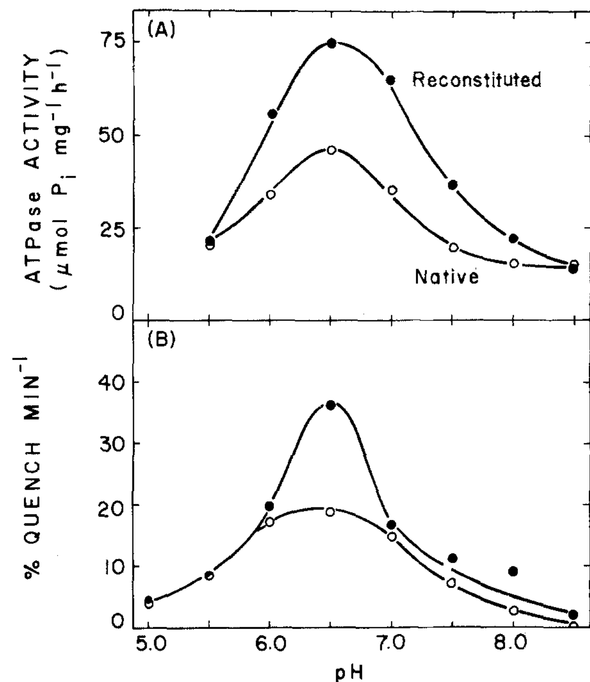

Fig. 2. Effect of pH on ATPase activity and H^+ transport associated with red beet plasma membrane (○) and reconstituted (●) H^+-ATPase. ATPase activity was assayed as described in Materials and Methods. H^+ transport was determined as the initial rate of fluorescence quenching (% quench/min) in a reaction mix containing 10 μM quinacrine, 0.5 μM valinomycin, 10 mM Tris/Mes, 50 mM KNO_3, 50 mM KCl and 5 mM $MgSO_4$. The transport assay was started by the addition of ATP to 5 mM final concentration

lated to the altered lipid environment of the enzyme. For subsequent assays, an assay temperature of 28°C was used for ATPase activity as well as H^+ transport since membrane vesicles above 30°C were leaky to protons (*unpublished results*).

Divalent Cation Stimulation

Both the native plasma membrane and reconstituted H^+-ATPases required a divalent cation for activity (Table 2). No ATPase activity was observed in the presence of ATP alone. Maximal activity was seen for both enzymes in the presence of Co^{2+} or Mg^{2+}. A lesser amount of activity was seen in the presence of Mn^{2+}, amounting to about 60% of the Mg^{2+}-stimulated level. This pattern of divalent cation specificity, in which $Co^{2+} > Mg^{2+} > Mn^{2+}$, is similar to that observed for the vanadate-sensitive ATPase of corn root plasma membrane (O'Neill et al., 1983) and for a partially purified corn root plasma membrane ATPase (DuPont, Burke & Spanswick, 1981). Overall these data indicate that a divalent cation is essential for enzyme activity for both H^+-ATPases and that the divalent cation requirements of the plasma membrane H^+-ATPase were unchanged by reconstitution.

Substrate Specificity

The substrate specificity of the native plasma membrane and reconstituted enzyme was investigated. Phosphohydrolase activity associated with the two enzymes is presented in Table 3. Clearly both ATPases utilized many substrates. However, none were as effective as ATP in catalyzing enzyme activity. Three nucleotide diphosphates (UDP, IDP, GDP) were utilized to some extent, with UDP the preferred nucleotide diphosphate. Ratios of UDPase/ATPase varied in separate experiments, suggesting variable contamination of the plasma membrane fraction by Golgi membranes rather than a Golgi origin for the H^+-ATPase of the plasma membrane fraction. This does not exclude the possibility that some of the H^+-ATPase in this plasma membrane fraction is derived from Golgi transition vesicles en route to the plasma membrane *in vivo*. The possible involvement of transition vesicles and the distribution of UDPase activity on continuous sucrose gradients of red beet microsomes was discussed in the accompanying paper (O'Neill & Spanswick, 1984).

From the data shown in Table 3, it can be seen that PNPase activity was minor and became negligi-

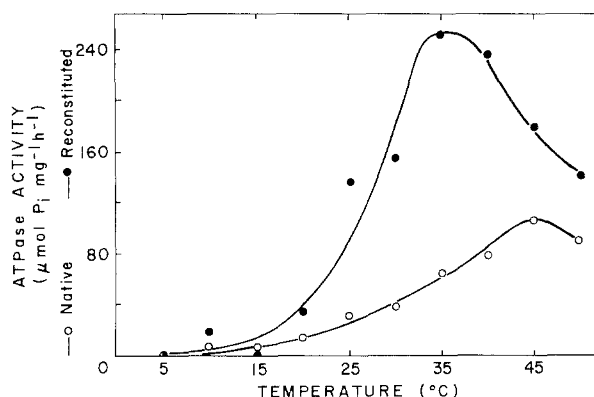

Fig. 3. Effect of temperature on ATPase activity associated with red beet plasma membrane (○) and reconstituted (●) H^+-ATPase. ATPase activity was assayed for 10 min as described in Materials and Methods

Table 2. Effect of divalent cations on ATPase activity of red beet plasma membrane and reconstituted ATPase

Divalent cation	ATPase activity[a]			
	Plasma membrane		Reconstituted ATPase	
	Specific activity (μmol P_i mg^{-1} hr^{-1})	% of Mg^{2+} stimulation	Specific activity (μmol P_i mg^{-1} hr^{-1})	% of Mg^{2+} stimulation
None added	0	0	0	0
$MgSO_4$	39.7	100%	83.0	100%
$MnSO_4$	22.5	57%	50.2	60%
$CoSO_4$	42.6	107%	84.2	101%
$ZnSO_4$	0.4	1%	3.3	4%
$CaSO_4$	3.0	7%	4.0	5%

[a] Total ATPase activity was determined in the presence of 5 mM ATP, 30 mM Tris/Mes, 50 mM KCl, 1 mM NaN_3, 2 μM gramicidin at pH 6.5 and 28°C. The divalent cation salt was present at a concentration of 5 mM.

Table 3. Phosphohydrolase activity associated with red beet plasma membrane and reconstituted ATPase

Substrate	Phosphohydrolase activity[a]			
	Plasma membrane		Reconstituted ATPase	
	Specific activity (μmol P_i mg^{-1} hr^{-1})	% of ATP level	Specific activity (μmol P_i mg^{-1} hr^{-1})	% of ATP level
ATP	40.97	100	145.43	100
UDP	11.06	27	42.34	29
IDP	5.21	13	21.67	15
GDP	4.05	10	21.95	15
PNP	4.11	10	3.10	2
UTP	2.18	5	11.74	8
ITP	1.73	4	7.87	5
GTP	1.43	3	14.43	10
AMP	0.86	2	0.25	0.2
ADP	0	0	6.99	4.8
CDP	0	0	2.95	2.0
CTP	0	0	6.94	4.8
PPi	0	0	9.30	6.4

[a] Phosphohydrolase activity was determined in the presence of 5 mM $MgSO_4$, 30 mM Tris/Mes, 50 mM KNO_3, 25 mM KCl, 1 mM NaN_3, 2 μM gramicidin at pH 6.5 and 28°C. Substrate was added to a final concentration of 5 mM.

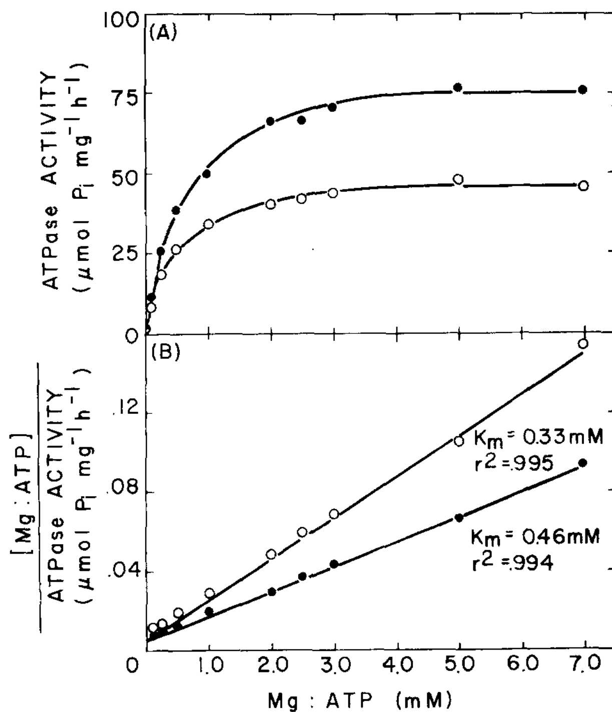

Fig. 4. Effect of Mg : ATP concentration on ATPase activity associated with the native plasma membrane (○) and reconstituted (●) H^+-ATPase of red beet (panel *A*). Assays were performed as described in Materials and Methods. Hanes-Woolf plot of the data in panel *A* for native (○) and reconstituted (●) ATPase activity (panel *B*). Lines were fit by regression analysis

Table 4. Proton translocating ATPase activity associated with red beet plasma membrane and reconstituted vesicles

Substrate (5 mM)	H^+ Transport[a]			
	Plasma membrane vesicles		Reconstituted vesicles	
	Initial rate of quench (% quench/min)	% of ATP	Initial rate of quench (% quench/min)	% of ATP
ATP : Mg	16.7	100	66.2	100
CTP : Mg	0	0	0.6	1.0
GTP : Mg	1.5	9	1.0	1.5
ITP : Mg	0	0	0.3	0.5
UTP : Mg	1.5	9	0	0
ADP : Mg	0	0	0	0
ATP	0	0	0	0

[a] H^+ transport was assayed by monitoring fluorescence quenching under assay conditions similar to the phosphohydrolase assay.

ble following reconstitution. Pyrophosphatase activity was increased following reconstitution, yet PP_i was not effective in catalyzing H^+ transport activity of the reconstituted enzyme (*not shown*).

With Mg^{2+} as the divalent cation, we next examined the substrate specificity of H^+ transport un-

der assay conditions identical to that of the phosphohydrolase assay except that gramicidin was not included in the reaction mix (Table 4). It is apparent that only ATP : Mg is effective in fueling H^+ transport for both the native plasma membrane and reconstituted enzymes. Transport activity in the presence of CTP, GTP, ITP or UTP (Mg^{2+} salts) was insignificant by comparison. The nucleotide diphosphates, GDP, IDP and UDP, did not support transport although these substrates were hydrolyzed to some extent (Table 3). Likewise, no H^+ transport was seen in the presence of ADP, or ATP with no Mg^{2+} present, as expected from the divalent cation results (Table 2). Two general conclusions can be reached. First, a divalent cation such as Mg^{2+} or Co^{2+} is required for activity of both the native plasma membrane and reconstituted H^+-ATPase, and second, both phosphohydrolase activity and H^+ transport preferentially utilize ATP : Mg as substrate. These two features are unchanged by solubilization and reconstitution and, therefore, are most likely intrinsic properties of the plant plasma membrane H^+-ATPase.

Mg : ATP Kinetics

The Mg : ATP kinetics of the native plasma membrane and reconstituted H^+-ATPase were examined under optimal conditions. Figure 4 shows the total ATPase activities of both enzymes (panel *A*) and a Hanes-Woolf plot of these data (panel *B*). Total ATPase activity for both enzymes saturates between 3 and 5 mM Mg : ATP and indicates simple Michaelis-Menten kinetics. The Hanes-Woolf transformation (panel *B*) of the data in panel *A* is linear for both enzymes, with a K_m of 0.33 and 0.46 mM Mg : ATP for native and reconstituted H^+-ATPase, respectively. The close agreement in the K_m values for the native and reconstituted enzymes indicates that the H^+-ATPase is not altered by the solubilization/reconstitution procedure with respect to Mg : ATP kinetics. These K_m values for the red beet native plasma membrane and reconstituted ATPase were similar to those reported by Briskin and Poole (1983*a*) for a red beet plasma membrane fraction (K_m = 0.52 mM Mg : ATP) and by Vara and Serrano (1982) for oat root plasma membrane ATPase (K_m = 0.33 mM Mg : ATP).

Monovalent Ion Stimulation

Figure 5 (panel *A*) shows the effects of various monovalent salts on H^+ transport of reconstituted vesicles. These quench assays were performed on an equal protein basis using a population of reconstituted vesicles that were strongly inhibited by vanadate (panel *B*). Transport activity of the reconstituted H^+-ATPase showed a pattern of salt stimulation that differed clearly from that of the H^+-ATPase of red beet tonoplast membranes and isolated vacuoles (Bennett et al., 1984). In the presence of either K_2SO_4 or choline chloride, only a small amount of H^+ transport occurred. Fluorescence quenching was enhanced greatly by KCl, with KBr having a similar effect.

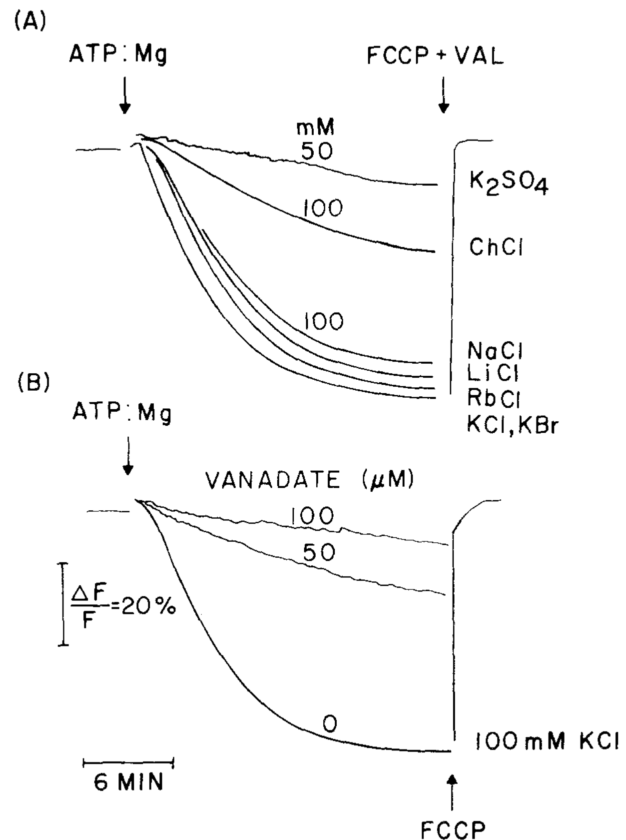

Fig. 5. Effect of monovalent ions on H^+ transport of reconstituted H^+-ATPase. Fluorescence quench assays were carried out on an equal protein basis (35 μg protein/quench assay) in (*A*) a basic reaction mix containing vesicles, 5μM acridine orange, and 10 mM Tris/Mes (pH 6.5), or (*B*) in the same reaction mix plus sodium orthovanadate (50 or 100 μM final concentration) and 100 mM KCl. Vesicles were preincubated with the appropriate salts as indicated (panel *A*). The assay was initiated by the addition of Mg : ATP to a final concentration of 5 mM. FCCP and/or valinomycin were added to a final concentration of 0.5 μM each where indicated

The pattern of monovalent ion stimulation seen in this series of quench assays suggests that (1) a cation is required with an order of preference such that $K^+ > Rb^+ > Li^+ > Na^+ >$ choline and (2) that an anion such as Cl^- or Br^- is also essential, with NO_3^- less effective (*see* Fig. 7). This latter require-

Table 5. Effect of monovalent cations on ATPase activity of red beet plasma membrane and reconstituted ATPase

Additions	ATPase activity[a]		
	Specific activity (μmol P_i mg^{-1} hr^{-1})	% Salt stimulation	% of KCl stimulation
Plasma membrane:			
$MgSO_4$	38.5	—	—
+KCl	48.5	26	100
+NH_4Cl	46.9	22	85
+RbCl	46.5	21	81
+NaCl	46.3	20	77
+CsCl	45.9	19	73
+LiCl	42.4	10	38
+Choline Cl	41.3	7	27
Reconstituted ATPase:			
$MgSO_4$	22.1	—	—
+KCl	72.4	228	100
+NH_4Cl	75.0	240	105
+RbCl	59.5	170	75
+NaCl	61.4	179	79
+CsCl	66.3	200	88
+LiCl	51.5	133	58
+Choline Cl	29.1	32	14

[a] ATPase activity was determined in the presence of 5 mM ATP, 5 mM $MgSO_4$, 30 mM Tris/Mes, 2 μM gramicidin, 0.5 μM valinomycin at pH 6.5 and 28°C and the added monovalent salt at a concentration of 50 mM.

ment for an anion may represent the need for a permeant anion which permits the formation of a pH gradient by dissipating the membrane potential through charge compensation. A second possibility is that anions may be involved in a direct activation of the ATPase as reported previously for corn root H^+-ATPase (Bennett & Spanswick, 1983*a*). It was not possible to distinguish between these two possibilities by looking at H^+ transport alone. Thus, the effect of various monovalent cations and anions on ATPase activity of the plasma membrane and reconstituted H^+-ATPases were next examined (Tables 5 and 6). Table 5 shows the effect of monovalent cations in total ATPase activity. Monovalent salts were added to a final concentration of 50 mM. Gramicidin and valinomycin were added to relieve the pH gradient and membrane potential, respectively, insuring maximal activity of the H^+-ATPase, and thus the ATPase activity results may not be strictly comparable with those of H^+ transport. For both the plasma membrane and reconstituted ATPases, all salts stimulated activity above the no monovalent salt level. The amount of salt stimulation was greatly enhanced following reconstitution, with KCl stimulation increasing from 26 to 228%. The increased salt stimulation probably reflects the purification that occurs during the solubilization/reconstitution procedure in that salts present in the plasma membrane fraction are removed from the solubilized enzyme extract during gel filtration (O'Neill & Spanswick, 1984). A corollary of this is that the level of ATPase activity seen in the absence of added monovalent salt for the plasma membrane is probably not a true estimate of the no salt level, instead representing activity in the presence of an unknown quantity of contaminating salts. The amount of KCl stimulation of plasma membrane ATPase activity reported here (26%) is similar to that reported previously by Briskin and Poole (1983*a*) for a red beet plasma membrane fraction of lighter density.

For the plasma membrane H^+-ATPase, a pattern of cation stimulation could be determined at a cation concentration of 50 mM (Table 5). Cation stimulation showed an order of preference of $K^+ > NH_4^+ > Rb^+ > Na^+ > Cs^+ > Li^+ >$ choline. Following reconstitution, the basic order of cation preference was similar with K^+, NH_4^+, Rb^+ and also Cs^+ the preferred cations. Choline was less stimulating while Li^+ was more stimulatory for the reconsti-

Table 6. Effect of monovalent anions on ATPase activity of red beet plasma membrane and reconstituted ATPase

Additions	ATPase activity[a]		
	Specific activity (μmol P_i mg^{-1} hr^{-1})	% Salt stimulation	% of KCl stimulation
Plasma membrane:			
$MgSO_4$	38.5	—	—
+KCl	48.5	26	100
+KBr	46.2	20	77
+K Acetate	45.9	19	73
+K Bicarbonate	44.3	15	58
+K_2SO_4	41.0	7	27
+KNO_3	40.9	6	23
+KI	40.1	4	15
+KSCN	12.2	−68	0
Reconstituted ATPase:			
$MgSO_4$	22.1	—	—
+KCl	72.4	228	100
+KBr	64.0	190	83
+K Acetate	64.5	193	85
+K Bicarbonate	74.1	236	104
+K_2SO_4	57.1	159	70
+KNO_3	59.5	170	75
+KI	57.5	161	71
+KSCN	20.6	−9	0

[a] ATPase activity was determined in the presence of 5 mM ATP, 5 mM $MgSO_4$, 30 mM Tris/Mes, 2 μM gramicidin, 0.5 μM valinomycin at pH 6.5 and 28°C and the added monovalent salt at a concentration of 50 mM.

tuted enzyme. These cation effects on ATPase activity are in basic agreement with the results seen in Fig. 5 for H^+ transport associated with reconstituted vesicles.

The effect of monovalent anion on ATPase activity of both enzymes was novel for a vanadate-sensitive H^+-ATPase derived from the plasma membrane (Table 6). The plasma membrane ATPase showed an order of anion preference in which $Cl^- > Br^- >$ acetate $> HCO_3^- > SO_4^{2-} > NO_3^- > I^-$. The chaotropic anion, SCN^-, was inhibitory at 50 mM final concentration. Similar inhibition by SCN^- was observed for both the NO_3^- and vanadate-sensitive ATPases of corn roots (O'Neill et al., 1983) and the H^+-ATPase of red beet tonoplast (Bennett et al., 1984). The reconstituted ATPase was also stimulated by anions to a greater extent than was the plasma membrane ATPase. The order of anion preference was modified by reconstitution in that $HCO_3^- > Cl^- > Br^- =$ acetate $> NO_3^- > I^- > SO_4^{2-}$. Stimulation by these anions cannot be due to dissipation of membrane potential because the assay was performed in the presence of gramicidin and valinomycin, which would serve to abolish both gradients of pH and membrane potential. Instead, these results suggest that anions may play a direct role in activating the H^+-ATPase by some direct stimulatory salt effect.

Cl^- Activation of the H^+-ATPase

That Cl^- is stimulating the H^+-ATPase of reconstituted vesicles can be seen in Fig. 6. In the absence of K^+ and/or Cl^-, but in the presence of 5 mM $MgSO_4$, only a small amount of transport was supported. When 10 mM Cl^- was supplied as 5 mM $MgCl_2$, a slight stimulation of quenching occurred. This was further stimulated upon the inclusion of 10 mM K^+ in the reaction mix (5 mM $MgSO_4$ + 10 mM KCl), suggesting a stimulation by the cation. Additional amounts of K^+ and Cl^- (25 and 100 mM final concentration) led to increased fluorescence quenching, and hence, H^+ transport. These anion effects agree with the earlier findings in Fig. 6 showing the salt effects on H^+ transport and with the

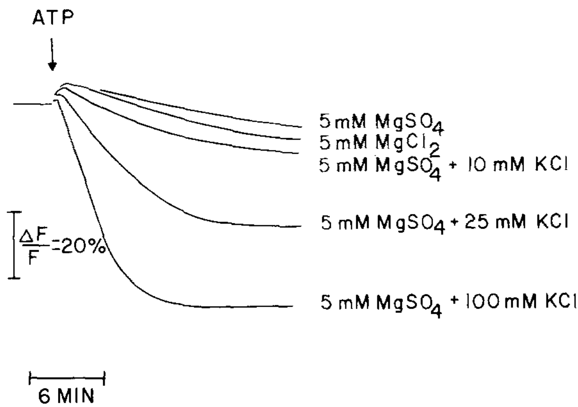

Fig. 6. Effect of anions on H^+ transport of reconstituted H^+-ATPase. Fluorescence quench assays were carried out on an equal protein basis (30 μg/quench assay) in a basic reaction mix containing vesicles, 5 μM acridine orange, 10 mM Tris/Mes (pH 6.5), and the various divalent and monovalent salts listed. The assay was initiated by the addition of ATP to a final concentration of 5 mM, equimolar with the Mg^{2+} already in the reaction mix

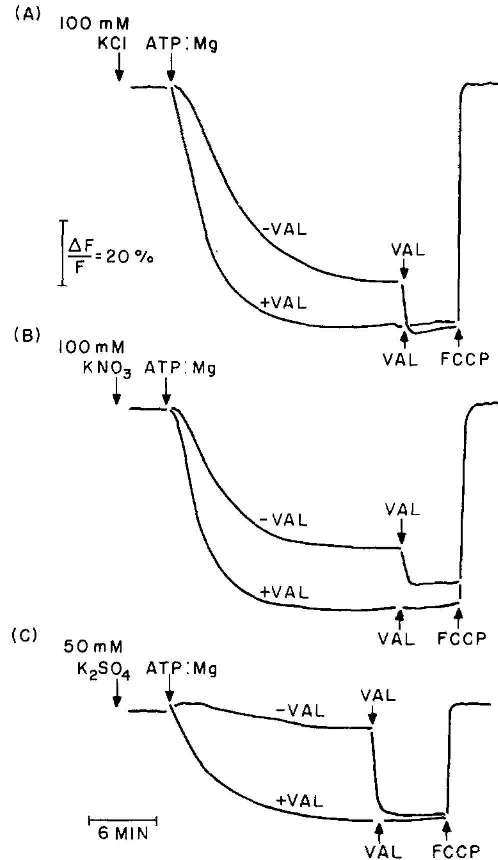

Fig. 7. Valinomycin effects on H^+ transport of reconstituted H^+-ATPase in the presence and absence of anions. Fluorescence quench assays were carried out on an equal protein basis (35 μg/quench assay) in a basic reaction mix containing vesicles, 5 μM acridine orange, 10 mM Tris/Mes (pH 6.5), and either (*A*) 100 mM KCl, (*B*) 100 mM KNO_3, or (*C*) 50 mM K_2SO_4. Valinomycin was added to the vesicle suspension to a final concentration of 0.5 μM either before or after initiating the assay with ATP:Mg (5 mM). FCCP was added where indicated to a final concentration of 0.5 μM

monovalent ion effects on ATPase activity presented in Tables 5 and 6.

Effect of Anions in the Presence of Valinomycin

From Figs. 5 and 6 it is apparent that H^+ transport activity is dependent on the presence of anions that may be directly activating the H^+-ATPase and/or acting as permeant anions. Maximal rates of transport also required the presence of valinomycin in addition to a permeant anion such as Cl^- or NO_3^-. Figure 7 presents H^+ transport assays using reconstituted vesicles in the presence of an anion and plus or minus valinomycin (0.5 μM). When vesicles were preincubated with 50 mM K_2SO_4, 100 mM KCl, or 100 mM KNO_3, fluorescence quenching was observed upon the addition of ATP:Mg. Quenching is greater in the presence of Cl^- or NO_3^-, two permeant anions, relative to that seen in the presence of SO_4^{2-}, an impermeant anion.

From this result alone, it could be concluded that Cl^- and NO_3^- are stimulating H^+ transport by acting as permeant anions, moving inward and dissipating a positive interior membrane potential. However, when valinomycin was added to the vesicles at the beginning or end of the fluorescence quench assay, an additional stimulation of quenching, and hence H^+ transport, occurred which was reversible by FCCP. This would suggest that valinomycin was acting in a stimulatory manner by providing a pathway for compensatory counter-ion movement and thereby dissipating the membrane potential. Apparently, 100 mM permeant anion is not adequate for dissipating an interior positive membrane potential in the reconstituted vesicle system. The valinomycin effect is reasonable, since K^+ present in the solubilization buffer may become sealed inside the reconstituted vesicles. This possibility was tested using a suspension of reconstituted vesicles incubated in the presence of acridine orange, but without ATP:Mg or K^+. When nigericin was added to the suspension, a proton gradient (interior acid) was established through the exchange of K^+ for H^+ by nigericin (*not shown*). This confirmed that K^+ was locked up inside sealed vesicles with a low passive permeability to this cation. Given these results, it seems likely that valinomycin is abolish-

ing the membrane potential by facilitating counter-ion movement rather than by delivering K^+ to the inside of the vesicle and actually stimulating ATPase activity by making a K^+/H^+ exchange by the enzyme possible.

Figure 8 diagrams the way we visualize the interaction between anions and valinomycin with the proton pump. The activity of the ATPase is proposed to be electrogenic involving the movement of H^+ into the vesicle interior. This electrogenic activity leads to the buildup of an interior positive membrane potential (Ψ), which exerts a back pressure on the H^+-ATPase, slowing down its H^+-translocating activity. Anions such as Cl^- or NO_3^- can stimulate H^+ transport by permeating the membrane and neutralizing some of the positive change. However, the dissipatory effect on the membrane potential by the permeant anion may be inadequate to relieve Ψ under certain conditions, especially if anions are also involved in direct activation of the pump. This would be the case if K^+ was locked up inside sealed vesicles that have low passive permeability to this cation. In this situation, valinomycin would facilitate counter-ion movement and in this manner could dissipate Ψ. An alternative explanation must also be considered. Valinomycin may also be delivering K^+ to the inside of the vesicles, stimulating the ATPase directly by functioning in K^+/H^+ exchange by the ATPase.

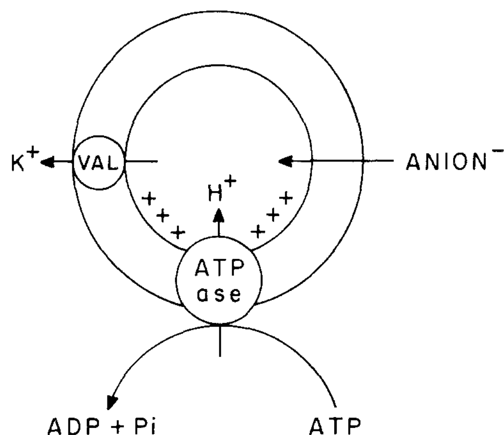

Fig. 8. Schematic model of a reconstituted H^+-ATPase vesicle illustrating the interaction between permanent anions and valinomycin with the proton pump. Positive charges inside the vesicle indicate the buildup of a positive interior membrane potential

Conclusions

In general, the characteristics of the solubilized and reconstituted red beet plasma membrane ATPase are similar to those of the ATPase associated with native plasma membranes. Those characteristics which have traditionally been used to identify the plant plasma membrane ATPase such as substrate specificity, pH optimum, inhibitor sensitivity, and cation stimulation were qualitatively the same in native and reconstituted vesicles indicating that they are intrinsic properties of the ATPase. Two quantitative differences observed following reconstitution were increased sensitivity to inhibition by vanadate and increased stimulation of activity by monovalent cations. Both results are similar to those observed for the *Neurospora* plasma membrane ATPase following purification (Bowman, Blasco & Slayman, 1981).

The characteristics of the red beet plasma membrane ATPase were similar to those of other plant plasma membrane ATPases and similar to the plasma membrane ATPase of *Neurospora* (Bowman & Slayman, 1977; Perlin & Slayman, 1982; Scarborough, 1980). One notable difference, however, was the stimulation of ATPase activity and H^+ transport by anions. This stimulation of activity did not result from anions acting solely as a permeant anion since anion stimulation was apparent under conditions where membrane potentials would not form (i.e., in the presence of valinomycin). The strong inhibition of the ATPase by vanadate (>90%) also indicated that the stimulation by anions did not result from contamination by tonoplast ATPase which is known to be anion-stimulated but insensitive to vanadate (Bennett & Spanswick, 1983*b*; Bennett et al., 1984). This result distinguishes the red beet H^+-ATPase from the reconstituted H^+-ATPase derived from an oat plasma membrane fraction (Vara & Serrano, 1982) whose H^+ transport was not shown to be inhibited by vanadate and was stimulated preferentially by NO_3^- over Cl^-. The fact that Vara and Serrano (1982) report valinomycin stimulation of H^+ transport in the presence of K_2SO_4, but no additional stimulation of H^+ transport by valinomycin in the presence of 100 mM KNO_3, suggests that in the oat reconstituted system anions are acting only as permeant anions, dissipating membrane potential.

For both the reconstituted oat and red beet plasma membrane ATPase it was observed that H^+ transport could occur in the absence of K^+ if a permeant anion was present (Cl^- or NO_3^-), although the addition of K^+ did stimulate H^+ transport. The K^+ stimulation of H^+ transport was greater for the red beet H^+-ATPase but, as with the reconstituted oat H^+-ATPase, the effect of K^+ addition on H^+ transport was immediate, indicating that K^+ was acting at the cytoplasmic face of the enzyme. This would not implicate a direct involvement of K^+ in

transport by the enzyme (i.e., K^+ participation in K^+/H^+ exchange). However, this result does not preclude the possibility that the H^+-ATPase catalyzes K^+/H^+ exchange of variable stoichiometry.

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