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Solubilization and Reconstitution of a Vanadate-Sensitive H^+-ATPase from the Plasma Membrane of *Beta vulgaris*

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Summary. A vanadate-sensitive H^+-translocating ATPase isolated from red beet plasma membrane has been solubilized in active form and successfully reconstituted into artificial proteoliposomes. The H^+-ATPase was solubilized in active form with deoxycholate, CHAPSO or octylglucoside in the presence of glycerol. Following detergent removal by gel filtration and reconstitution into proteoliposomes, ATP:Mg-dependent H^+ transport could be measured as ionophore-reversible quenching of acridine orange fluorescence. Solubilization resulted in a three- to four-fold purification of the plasma membrane ATPase, with some additional enrichment of specific activity following reconstitution. H^+ transport activity was inhibited half-maximally between 1 and 5 μM vanadate (Na_3VO_4) and nearly abolished by 100 μM vanadate. ATPase activity of native plasma membrane showed a K_i for vanadate inhibition of 9.5 μM, and was inhibited up to 80% by 15 to 20 μM vanadate (Na_3VO_4). ATPase activity of the reconstituted vesicles showed a K_i of 2.6 μM for vanadate inhibition. The strong inhibition by low concentrations of vanadate indicates a plasma membrane rather than a mitochondrial or tonoplast origin for the reconstituted enzyme.

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

Introduction

Based on electrophysiological evidence, the presence of an electrogenic H^+-translocating ATPase in the plasma membrane of *Neurospora* (Slayman, 1965*a*,*b*) and giant algal cells (Kitasato, 1968; Spanswick, 1972) was proposed. The existence of such an H^+-ATPase has been confirmed biochemically for *Neurospora* (Scarborough, 1980; Perlin & Slayman, 1982). A similar biochemical confirmation for an H^+-ATPase in the plasma membrane of plant cells has not been conclusive, although the idea of a plasma membrane H^+-ATPase for plant cells in general has gained wide support. The major obstacle in identifying the plant plasma membrane H^+-ATPase has been the inability to obtain plasma membrane preparations from plant tissue uncontaminated by other cellular membranes.

Recently, an H^+-ATPase from cultured tobacco cells has been identified and reported to be of plasma membrane origin (Sze & Churchill, 1981). However, this H^+-ATPase had characteristics which are now thought to belong to a tonoplast H^+-ATPase (Sze, 1982). More recently, Vara and Serrano (1982) have reconstituted an H^+-translocating ATPase associated with a plasma membrane fraction from oat roots. The properties of this ATPase were clearly different from that of the tonoplast ATPase (Walker & Leigh, 1981; Bennett & Spanswick, 1983*a*; O'Neill, Bennett & Spanswick, 1983; Bennett, O'Neill & Spanswick, 1984). However, it was not conclusively shown that the H^+ transport activity of the reconstituted enzyme was not derived from mitochondrial membranes, which are known to contaminate plasma membrane preparations from oat roots (Leonard & Hodges, 1973) and other plant tissue.

A major effort in our laboratory for several years had been to demonstrate the activity of the proposed H^+-translocating ATPase of the plant plasma membrane *in vitro* and identify the ions transported. We have recently been able to demonstrate the existence of two distinct H^+-translocating ATPases associated with the plasma membrane and tonoplast of red beet storage tissue (Bennett et al., 1984). These two H^+-ATPases were separated effectively on discontinuous sucrose gradients and identified by their differential sensitivity to inhibition by NO_3^- and vanadate. The tonoplast ATPase, equilibrating at a low density (1.09 g/cc), was greatly stimulated by gramicidin, was stimulated preferentially by anions rather than cations, and was strongly inhibited by NO_3^-. We have previously shown that NO_3^- inhibition of anion-sensitive H^+-ATPase activity in corn root membranes can be used as a means of identifying low density tonoplast H^+-ATPase (O'Neill et al., 1983).

The plasma membrane ATPase of red beet

equilibrated at higher densities (1.16 to 1.18 g/cc) and was relatively less stimulated by gramicidin. Both the ATPase activity and H^+ transport of the plasma membrane fraction, however, were largely insensitive to inhibition by NO_3^- and instead were strongly inhibited by low concentrations of vanadate. The existence of this higher density, vanadate-sensitive H^+-ATPase associated with a plasma membrane fraction (Bennett et al., 1984) was a promising finding, especially since we had been unable to show H^+ transport associated with plasma membranes from corn (Perlin & Spanswick, 1982; Spanswick & Bennett, 1983).

The finding that H^+ transport activity associated with the plasma membrane fraction was insensitive to NO_3^-, but inhibited by vanadate, was our first indication that this H^+-ATPase was perhaps the elusive H^+-translocating ATPase of the plant cell plasma membrane. We were first able to observe vanadate inhibition by blocking H^+ transport activity of the contaminating tonoplast pump with 100 mM KNO_3. In the presence of NO_3^-, the activity of the plasma membrane pump was more apparent, and vanadate inhibition of H^+ transport (up to 80% with 100 μM vanadate) was revealed (Bennett et al., 1984).

The transport activity of the plasma membrane vesicles was not as high as that of the tonoplast H^+-ATPase when determined on an equal protein basis. So, in order to restore higher rates of H^+ transport and to enrich the vanadate-sensitive, NO_3^--insensitive H^+-ATPase, we chose to solubilize the plasma membrane ATPase and reconstitute the solubilized enzyme into sealed lipid vesicles permitting the formation of pH gradients which could be measured *in vitro*.

In this report, we describe the isolation, solubilization, and reconstitution of the red beet plasma membrane H^+-ATPase. Details of the response of both the native plasma membrane and reconstituted H^+-ATPase to inhibition by vanadate are also presented. A complete characterization of the reconstituted H^+-ATPase and its comparison with the native plasma membrane H^+-ATPase is presented in an accompanying paper (O'Neill & Spanswick, 1984).

Materials and Methods

Plant Material

Freshly harvested red beets (*Beta vulgaris* L.) were used for all membrane preparations. Following removal of the leafy tops, the beet storage tissue (swollen hypocotyl) was stored in moist vermiculite at 4°C in the dark until use, but not for more than one month.

Membrane Isolation

Microsomal membranes were prepared from red beet storage tissue as described previously by Briskin and Poole (1983*a*) with several modifications found to be important in maintenance of H^+ transport activity. Approximately 100 g fresh weight of red beet storage tissue were peeled, sliced and homogenized for 30 sec in a prechilled blender equipped with standard blades in 180 ml of (in mM): 250 sucrose, 70 Tris-Cl (pH 8.0), 4 DTT, 3 EDTA, 0.1% BSA and 0.5% polyvinylpyrrolidine (PVP-40). The homogenate was rapidly transferred to a second prechilled blender in which the standard blades had been replaced with double-edged razor blades for an additional 30 sec of homogenization. The homogenate was filtered through four layers of cheesecloth. The filtrate was made up to approximately 200 ml and then centrifuged for 10 min at 13,000 × *g* in a Beckman SW 27 rotor. Pelletable material was discarded. The supernatant was centrifuged for 30 min at 80,000 × *g* to pellet the microsomes, which were then resuspended in 250 mM sucrose, 5 mM Tris/Mes (pH 6.5) and 2 mM DTT. This procedure was repeated twice, and the resuspended microsomal membranes were combined. The combined microsomes were washed with an equal volume of (mM): 250 sucrose, 5 Tris/Mes (pH 6.5), 2 DTT and 50 KI, with continuous stirring. These membranes were repelleted for 30 min at 80,000 × *g* and the KI-washed microsomal pellets were resuspended in 250 mM sucrose, 5 mM Tris/Mes (pH 6.5), and 2 mM DTT.

The resuspended microsomes were then layered onto 16/26/34/38% (wt/wt) discontinuous sucrose gradients and centrifuged for 2 hr at 80,000 × *g* in a Beckman SW 27 rotor. All sucrose solutions contained 2 mM DTT and 5 mM Tris/Mes (pH 6.5). Membranes collecting at the 34/38% (wt/wt) interface were substantially enriched in vanadate-sensitive ATPase activity and glucan synthetase II activity, both proposed plasma membrane markers (Bennett et al., 1984). The 34/38% (wt/wt) interface membranes will be referred to as the native plasma membranes through this report. The 34/38% (wt/wt) interface fraction was collected, diluted with sucrose-free buffer, and repelleted for 30 min at 80,000 × *g*. The final pellet was resuspended in 45% glycerol, 1 mM DTT, 2 mM EDTA, 25 mM Tris/Mes (pH 7.5), with or without 150 mM KCl or 75 mM K_2SO_4. Resuspended plasma membranes were pinkish-red in color due to the presence of red beet betacyanin pigment trapped inside sealed membrane vesicles. The final yield of plasma membrane was between 1.5 and 3.0 mg protein per complete membrane preparation. Membrane samples were frozen in liquid N_2 and stored at −70°C until use, usually within 7 days. Continuous sucrose gradients (12 to 45%, wt/wt) were prepared as described previously (Bennett et al., 1984) using microsomes isolated from freshly harvested beets.

Solubilization

Three detergents, CHAPSO, deoxycholate and octylglucoside, were used for solubilization experiments. CHAPSO and octylglucoside were kept as buffered 15% aqueous stocks refrigerated or at room temperature. Deoxycholate (DOC) was purified by filtration through activated charcoal and recrystallization from ethanol as described by MacLennan (1970) prior to prepara-

tion of a 10% stock solution (pH 8.0 with NaOH) which was stored at room temperature. Detergent titrations were performed on membrane samples by adding with constant stirring a detergent stock at twice the desired final detergent concentration in a 1 : 1 ratio with the membrane sample. The final concentration of membrane protein was 1.5 mg/ml. Following a 10-min incubation at 4°C, the detergent-membrane samples were centrifuged for 45 min at 180,000 × *g* in a Beckman Type 50 rotor. The supernatant and pellet were collected separately and assayed for ATPase activity and protein content. ATPase activities and proteins remaining in the supernatant fraction following centrifugation were considered to be solubilized. Pellets were resuspended in solubilization buffer and homogenized with a hand-held Teflon/glass homogenizer.

In the final solubilization procedure used for reconstitution, deoxycholate was added as a 10% stock solution to achieve the desired final detergent/protein ratio, and centrifugation was carried out as above. Optimal solubilization was achieved when membrane samples were resuspended to a final protein concentration of 1.5 mg/ml and deoxycholate was added to a final concentration of 0.3% (wt/vol) resulting in an overall detergent/protein ratio (wt/wt) of 2. The resulting supernatant containing the solubilized enzyme extract was collected for reconstitution.

Reconstitution

Soybean phospholipid in the form of sonicated liposomes was added in a 1 : 1 ratio to the solubilized enzyme extract and incubated for 10 min. The final concentration of phospholipid was 10 mg/ml. The DOC concentration was maintained at 0.3% by the addition of detergent to the phospholipid prior to mixing with the enzyme sample or during sonication. Following incubation of the solubilized enzyme-phospholipid-DOC mix, the detergent was removed by gel filtration through Sephadex G-200 (Pharmacia). A 1.6 × 30 cm column (Amicon) of Sephadex G-200 (23 cm bed height) was pre-equilibrated with elution buffer (2.5 mM Tris/Mes, 1 mM DTT at pH 6.5) and maintained at 20°C with a water jacket and circulating water bath.

Before applying the sample, the column was preloaded with 1 ml of 5 mg/ml sonicated phospholipid which was discarded routinely as the first turbid void volume fraction. A 2 to 3 ml aliquot of the sample was then layered onto the Sephadex column and eluted at a flow rate of 0.5 ml/min with elution buffer. The cloudy void volume containing the reconstituted proteoliposomes was then collected, diluted fourfold to remove residual DOC, and repelleted at 180,000 × *g* for 60 min. The final pellet was resuspended in 2.5 mM Tris/Mes (pH 6.5), 1 mM DTT plus or minus 10% glycerol to a final volume of approximately 1 to 1.5 ml. The reconstituted proteoliposomes were assayed immediately for transport and ATPase activity. Maintenance of reconstituted enzyme activity following freezing and thawing required the presence of either 10% glycerol or 250 mM sucrose.

Fractionation of the Sephadex G-200 column was carried out using an ISCO Fraction Collector (model 1850) equipped with a UV absorbance monitor. Successive fractions of 2 ml each were collected until all red beet pigment had passed through the column. The presence of red beet pigment was followed by measuring absorbance at 550 nm, the peak absorbance for betacyanins.

Transport Assays

The formation of interior acid pH gradients across vesicle membranes in response to the activity of a H^+-translocating ATPase was monitored as quenching of fluorescence of the permeant amine dye, quinacrine or acridine orange. Plasma membrane vesicles or reconstituted proteoliposomes were added to a reaction mix containing 10 mM Tris/Mes (pH 6.5), appropriate salts and/or inhibitors, and either 10 μM quinacrine or 5 μM acridine orange. Fluorescence quenching assays were initiated by the addition of ATP : Mg or ATP to a final concentration of 5 mM. The reaction volume was 1.5 ml. Fluorescence was measured at 28°C with a Perkin-Elmer 650-10S fluorescence spectrophotometer at excitation/emission wavelengths of 425/500 nm for quinacrine and of 472/525 nm for acridine orange. Approximately 70 to 100 or 35 μg of membrane protein were added per transport assay for native plasma membrane or reconstituted proteoliposomes, respectively.

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 or 38°C. The basic reaction mix contained 5 mM ATP/Tris (pH 6.5), 5 mM $MgSO_4$, 30 mM Tris/Mes (pH 6.5), and appropriate salts and/or inhibitors and ionophores in a final volume of 0.5 ml. The pH of the final reaction mix was adjusted to pH 6.5 at the appropriate assay temperature. The amount of membrane protein routinely used was 1.0 to 3.5 μg per assay.

For determining the effect of vanadate on ATPase activity. the inhibitor was diluted tenfold into the reaction mix to achieve the appropriate final concentration. Neither the pH of the reaction mix nor the enzyme blank absorbance values were significantly altered by the presence of vanadate when supplied as either sodium orthovanadate (Na_3VO_4) or vanadium oxide (V_2O_5). Vanadate did not interfere with color development of the Ames reagent although it has been reported to interfere with other methods of determining ATPase activity (Bowman & Slayman, 1979; Gallagher & Leonard, 1982).

UDPase activity was determined by measuring the release of inorganic phosphate from UDP (sodium salt) in a manner analogous to the ATPase assay. The reaction mix contained 1.5 mM UDP, 1.5 mM $MnSO_4$, 30 mM Tris/Mes (pH 7.0), and 0.1% Triton X-100. Activity was assayed for 30 min at 25°C. For both the ATPase and UDPase assays, 1.5% SDS was present in the Ames reagent (1% final in assay) to eliminate detergent interference (Peterson, 1978).

Protein Determination

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

Phospholipids

Purified mixed soybean phospholipid (L-α-phosphatidylcholine, Type IV-S, Sigma) containing approximately 40% phosphatidylcholine was used in the reconstitution procedure. This particular grade of soybean phospholipid is not contaminated with phosphate or protein that would interfere with ATPase assays and protein determinations, respectively. A 50 mg/ml stock of soybean phospholipid was prepared daily in 2.5 mM Tris/Mes (pH 6.5) and 1 mM DTT, sonicated to near clarity in a bath sonicator, and diluted to the desired final concentration.

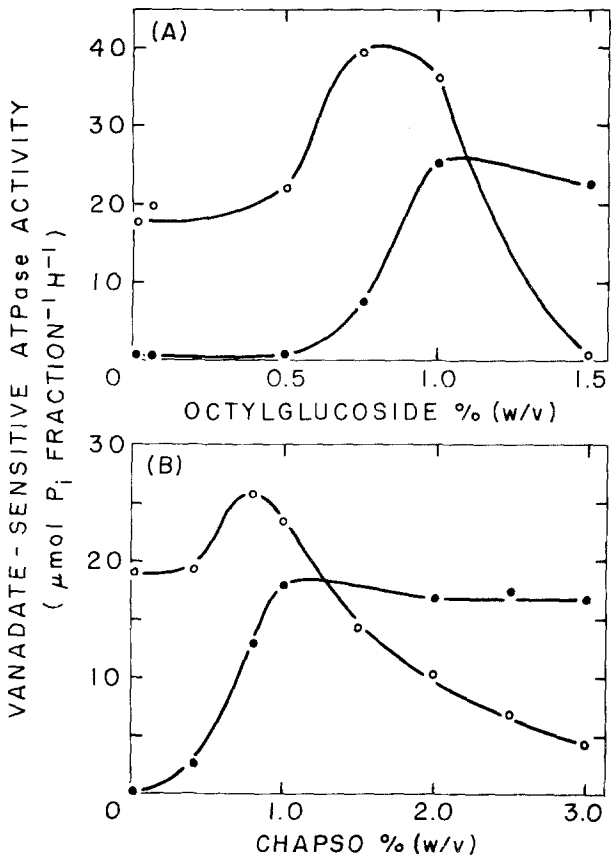

Fig. 1. Solubilization of plasma membrane ATPase activity with increasing concentrations of (*A*) octylglucoside and (*B*) CHAPSO. The amount of vanadate-sensitive ATPase activity remaining in the pellet (○) and the appearance of ATPase activity in the supernatant (●) are shown for both detergent titrations. Plasma membranes collected from a 34/38% interface were suspended at a protein concentration of 1.5 mg/ml in solubilization buffer containing 45% glycerol as described in Materials and Methods. ATPase activity was determined in the presence of (in mM): 5 ATP, 5 $MgSO_4$, 30 Tris/Mes, 1 NaN_3, and 50 KCl at pH 6.5 and 28°C. Vanadate-sensitive ATPase activity was calculated as the difference in total ATPase activity achieved in the presence or absence of 20 μM vanadate

Source of Chemicals

ATP was obtained from Boehringer Mannheim as the disodium salt and converted to the Tris salt by ion exchange chromatography 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 determined by Cantley et al. (1977). The ionophores and detergents were obtained from Calbiochem-Behring. All other chemicals were obtained from Sigma.

Results and Discussion

Solubilization

As a first step in reconstitution of the H^+-ATPase of the plasma membrane of red beet, a variety of detergents were screened for their effectiveness in solubilizing the enzyme in active form. ATPase activity in the absence and presence of 20 μM vanadate was monitored as a function of increasing concentration of detergent, and the vanadate-sensitive ATPase activity was calculated as the difference between the two. Sensitivity of the enzyme activity to vanadate was used here to identify only that activity belonging to the plasma membrane ATPase. The ATPase activity associated with the plasma membrane fraction from red beets is inhibited routinely up to 80% by 15 to 20 μM vanadate.

Detergent titrations using octylglucoside and CHAPSO are presented in Fig. 1. In Panel *A*, solubilization of vanadate-sensitive ATPase activity by octylglucoside was maximal at approximately 1.0% (wt/vol) at a protein concentration of 1.5 mg/ml. At this point, about 50% of the total vanadate-sensitive ATPase activity was recoverable in the supernatant, and approximately 40% of the protein had been solubilized. The release of total ATPase activity was similar to that of vanadate-sensitive ATPase (*data not shown*). Both total and vanadate-sensitive ATPase activities showed a release of latent activity in the pellet by the detergent between 0.5 and 1% octylglucoside (wt/vol). Higher amounts of octylglucoside released additional amounts of vanadate-sensitive ATPase activity from the pellet; however, this activity was not recovered in the supernatant, apparently reflecting inactivation of the plasma membrane ATPase at high concentrations of this detergent.

Figure 1*B* shows the effectiveness of a second detergent, CHAPSO, in solubilizing the plasma membrane H^+-ATPase in active form. Solubilization was maximal with 1% CHAPSO (wt/vol) at a protein concentration of 1.5 mg/ml. As with octylglucoside, higher concentrations of detergent released additional activity from the pellet that was not recovered in the supernatant. Unlike octylglucoside, though, which continued to solubilize membrane protein above 1% (wt/vol), CHAPSO above 1% (wt/vol) failed to release any additional protein to the supernatant (*data not shown*).

We also looked at the effect of the bile salt detergent, deoxycholate (DOC), on solubilization of the plasma membrane ATPase. This detergent has

been used successfully in the solubilization of many major ion translocating ATPases, including the Na^+/K^+-ATPase of animal cell plasma membrane (Kyte, 1971), the H^+-ATPase of *Neurospora* plasma membrane (Bowman, Blasco & Slayman, 1981; Perlin & Slayman, 1982), and more recently, the anion-sensitive H^+-ATPase of corn root microsomes (Bennett & Spanswick, 1983*b*). Figure 2 shows the effect of increasing concentrations of DOC on solubilization of vanadate-sensitive ATPase activity and protein, panels *A* and *B*, respectively. Given that the effectiveness of DOC in disrupting protein-phospholipid interaction is increased by the presence of a stabilizing counterion (Kyte, 1971; Helenius & Simons, 1975), solubilization with DOC was carried out in the presence of 0.15 M KCl. When membranes were resuspended to a protein concentration of 1.5 mg/ml, solubilization by DOC was maximal at 0.3% (wt/vol). This amounts to an overall optimal detergent/protein ratio of 2. At 0.3% DOC (wt/vol) there is a substantial increase in summed ATPase activity in the pellet and supernatant, again suggesting the release of considerable latent activity.

Additional DOC above 0.3% (wt/vol) failed to release additional activity to the supernatant. Problems of inactivation seen with octylglucoside were probably related to its effectiveness in solubilizing the enzyme. DOC, on the other hand, was effective only to a certain point and then additional detergent was without solubilizing effect. The beneficial corollary of this lack of efficiency is that DOC is not as inactivating for the enzyme should a critical ratio of detergent/protein be exceeded as appeared to be the case occasionally with octylglucoside. Vanadate-sensitive ATPase activity remained high in the supernatant even at 0.6% DOC (wt/vol). At the optimal detergent/protein ratio of 2, or at 0.3% DOC, it can be seen (Fig. 2*B*) that between 50 and 60% of the protein was released to the supernatant while 100% of the vanadate-sensitive ATPase originally associated with the membrane was solubilized. This apparent purification during solubilization, however, is confounded by the release of cryptic ATPase activity presumably from sites inside originally sealed vesicles. When solubilization with DOC was carried out in the absence of KCl (*not shown*), maximal solubilization did not occur until higher concentrations of detergent and the activity released was never as great as that seen with KCl present. Although comparable solubilization was obtained with either DOC or CHAPSO, further work was carried out using only DOC because of its demonstrated usefulness in reconstitution of several ion-translocating ATPases (Perlin & Slayman, 1982; Bennett & Spanswick, 1983*b*).

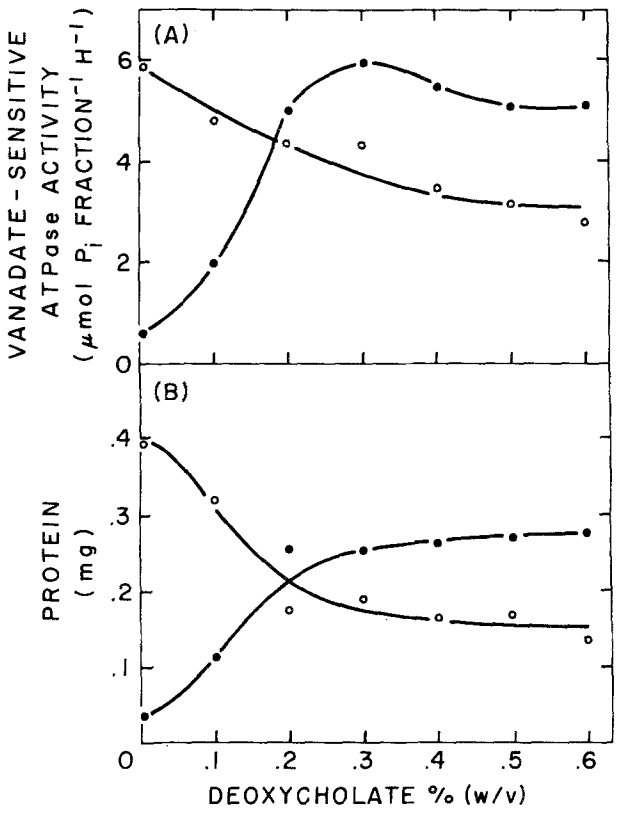

Fig. 2. Solubilization of plasma membrane ATPase activity and protein with increasing concentrations of deoxycholate. Panel *A* shows the release of vanadate-sensitive ATPase activity from the pellet (○) to the supernatant (●). Panel *B* shows the release of protein from the pellet (○) to the supernatant (●). Assay conditions were as described in the legend of Fig. 1 and as in Materials and Methods

The detergent titrations discussed thus far were all performed with 45% glycerol in the solubilization buffer. The presence of glycerol during detergent solubilization appears to be a critical factor determining whether or not an enzyme will be active following solubilization from the lipid bilayer (Kyte, 1971; Dean & Tanford, 1978; Bowman et al., 1981). Glycerol stabilizes the enzyme during solubilization, preventing inactivation. Earlier attempts to solubilize the plasma membrane ATPase in plants often resulted in enzyme inactivation or partial loss of activity (DuPont & Leonard, 1980; Brauer, Teel & Frick, 1982) perhaps because these solubilizations were not carried out in the presence of glycerol. More recently, attempts have been made to solubilize plant ATPases in the presence of glycerol

and these have been successful in obtaining a solubilized enzyme extract of high activity (Vara & Serrano, 1982; Bennett & Spanswick, 1983*b*).

In order to determine an optimal concentration of glycerol for solubilizing the vanadate-sensitive ATPase of red beet plasma membrane, we examined the effect of glycerol concentration on the maintenance of ATPase activity in the solubilized state. Table 1 shows the amount of both total and vanadate-sensitive ATPase activity found in the supernatant following solubilization with DOC. Clearly, the presence of glycerol was essential for the maintenance of both total and vanadate-sensitive ATPase activity after detergent solubilization. Maximal activity was maintained above 40% glycerol. Glycerol did not, however, significantly affect the extent of solubilization of membrane protein since release of protein into the supernatant was basically similar for all treatments (*not shown*). This also indicates that glycerol is not interfering significantly with sedimentation of solubilized and/or membrane-bound protein during the centrifugation step. Instead, the effect of glycerol appears to be related to its ability to provide a semi-hydrophobic environment for the solubilized enzyme.

From these results, a concentration of 45% glycerol (vol/vol) was chosen for solubilization. Following preliminary detergent and glycerol titrations, a routine solubilization scheme was developed with details given in Materials and Methods. This procedure yielded a solubilized enzyme extract, enriched between three- and fourfold in vanadate-sensitive ATPase activity, and suitable for reconstitution into artificial proteoliposomes.

Table 1. Effect of glycerol on the maintenance of ATPase activity following solubilization with deoxycholate[a]

% Glycerol (vol/vol)	ATPase activity (μmol P_i fraction^{-1} h^{-1}) Total	+Vanadate	Δ Vanadate[b]
0	2.07	1.37	0.70
10	4.70	2.54	2.16
20	8.45	3.72	4.73
30	17.05	7.42	9.63
40	27.45	13.20	14.25
60	25.19	12.57	12.62

[a] Equal aliquots of plasma membrane were pelleted and resuspended in solubilization buffer containing 0 to 60% glycerol at a protein concentration of 1.5 mg/ml. Deoxycholate was added to 0.3%. ATPase activities were measured in the supernatant fractions following centrifugation described in Materials and Methods.

[b] Vanadate-sensitive ATPase activity was calculated as the difference in total ATPase activity achieved in the presence or absence of 20 μM vanadate.

Reconstitution

While optimizing our reconstitution procedure, we examined the effects of various salts on the effectiveness of the reconstitution procedure. Overall, reconstitution was optimal when K^+, supplied as either KCl or K_2SO_4, was present in the solubilization buffer. It was not necessary to have K^+ in the elution buffer. Other compounds tested that were not essential for reconstitution of an active H^+-ATPase were Na_2ATP, EDTA and $MgSO_4$. In the case of $MgSO_4$, reconstitution was poor when $MgSO_4$ was added to the sample to a final concentration of 25 mM. Samples were routinely eluted with 2.5 mM Tris/Mes, 1 mM DTT at pH 6.5. Glycerol was not present in the elution buffer because it interfered with reconstitution.

The reconstitution procedure was monitored by collecting successive fractions during elution of the Sephadex G-200 column. A typical elution profile of ATPase activity, protein, and soluble beet pigment during column reconstitution is presented in Fig. 3. The elution of protein occurs over a relatively narrow range between fractions 8 and 13 when measured as OD_{280} or quantitatively (*see* Materials and Methods for protein determination). The distribution of protein is clearly separated from that of the soluble red beet pigment which eluted at a much slower rate. The peak protein fractions contained the peak ATPase and transport activities, indicating

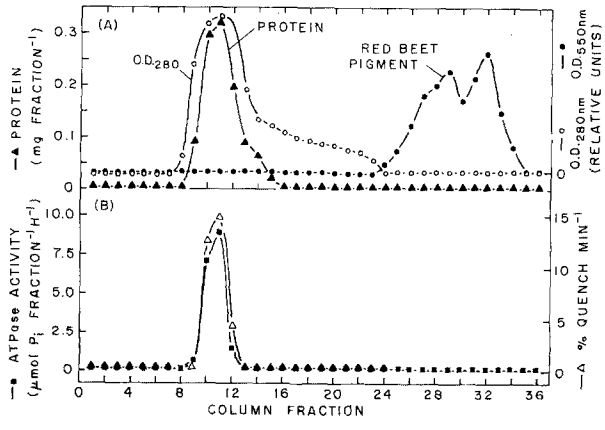

Fig. 3. Elution profile for the reconstituted ATPase during column fractionation. Panel *A* shows the distribution of protein (▲), $O.D._{280nm}$ (○), and $O.D._{550nm}$ (●). Panel *B* shows the distribution of ATPase activity (■) and H^+ transport activity (△) measured as initial rates using acridine orange. Details are given in Materials and Methods

that protein eluting in this region was reconstituted H^+-ATPase.

The enrichment of the plasma membrane ATPase that occurs during the solubilization-reconstitution procedure can be seen by following purification of the enzyme. A representative purification is presented in Table 2 showing the yield of enzyme units and the enrichment of specific activity following each step in the overall procedure. The most notable feature of the procedure is that most of the purification or enrichment of specific activity occurs during the initial solubilization step. Generally, the specific activity when assayed at 28°C for native plasma membranes was between 30 and 50 μmol P_i mg^{-1} h^{-1} and that of the reconstituted ATPase was between 80 and 145 μmol P_i mg^{-1} h^{-1}.

Some additional purification may occur during the column reconstitution step in that some contaminating soluble proteins may fail to associate with the phospholipids. An additional small amount of protein may also be lost during the final repelleting stage although this has not been significant when we have assessed protein loss during this step. Overall, the purification in Table 2 is nearly threefold, with a fairly good recovery (51%) of starting plasma membrane enzyme units. This amounts to a 25% recovery if latent ATPase of the native plasma membranes is also taken into account.

Transport Activity of the Reconstituted H^+-ATPase

Following reconstitution, the reconstituted vesicles were assayed for transport activity (Fig. 4). A suspension of membrane vesicles was incubated in the presence of $MgSO_4$, appropriate salts, and the fluorescent amine dye, acridine orange. After recording a stable baseline, ATP was added. There is an initial interaction between ATP and the dye, amounting to about 15% of the total fluorescence. This ATP-dye interaction is corrected in subsequent quench curves. Following the initial ATP-dye interaction, there was a progressive quenching of fluorescence, which reflected the acidification by the H^+-ATPase of the vesicle interior.

After 10 min, the fluorescence leveled off. Over the same time period and up to 30 min, ATPase activity (Fig. 4) assayed under similar conditions was constant, indicating that this leveling off of fluorescence was not due to a slowing down of the H^+ pump. Instead, the leveling off reflects a steady-

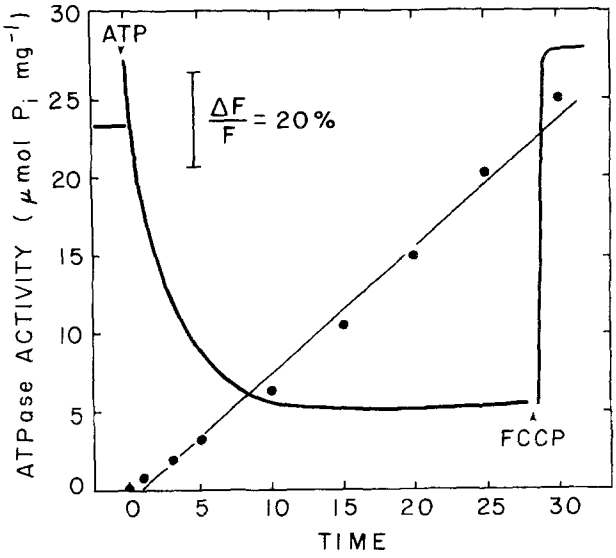

Fig. 4. Time course of ATPase activity (●) and H^+ transport activity (bold line) of reconstituted vesicles. ATPase was assayed as in Materials and Methods. For fluorescence quenching, vesicles were incubated with 5 mM $MgSO_4$, 50 mM KCl, 0.5 μM valinomycin, and 5 μM acridine orange. ATP and FCCP were added where indicated to final concentrations of 5 mM and 0.5 μM, respectively

Table 2. Purification of ATPase from the plasma membrane of red beet storage tissue

Fraction	Volume (ml)	Enzyme[a] units (total μmol P_i hr^{-1})	Yield[b] (%)	Protein (mg)	Yield[b] (%)	Specific activity (units mg^{-1})	Purification[c]
Plasma membranes	1.80	95.40	100	3.31	100	28.8	—
DOC-solubilized extract	1.58	121.34	128	1.66	50	73.3	2.5
DOC-insoluble pellet	1.50	84.96	89	1.31	40	61.2	—
Reconstituted proteoliposomes	1.20	48.83	51	0.58	17.5	84.0	2.9

[a] Enzyme unit = that amount of enzyme capable of hydrolyzing 1 μmol P_i h^{-1}.

[b] Yield is the % recovery of the initial starting plasma membrane values corrected for dilutions and aliquots removed for ATPase and protein determinations.

[c] Purification is based on specific activity.

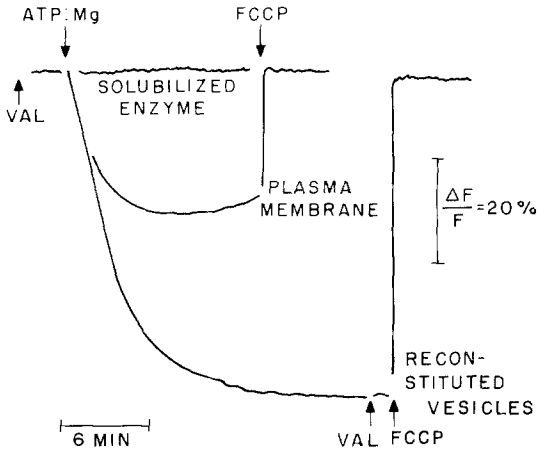

Fig. 5. Enhancement of ATP-dependent H^+ transport activity following solubilization. Transport assays were performed on native, solubilized and reconstituted vesicles on an equal protein basis (60 μg protein/quench) and in the presence of 50 mM KNO_3, 50 mM KCl, 0.5 μM valinomycin, and 5 μM acridine orange. ATP : Mg and FCCP were added to final concentrations of 5 mM and 0.5 μM, respectively

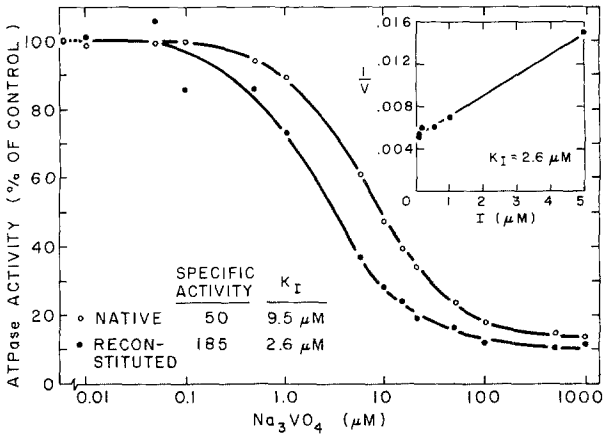

Fig. 6. Inhibition of ATPase activity by vanadate. Control activities were 50 μmol P_i mg^{-1} h^{-1} for native plasma membranes (○) and 185 μmol P_i mg^{-1} h^{-1} for reconstituted vesicles (●). ATPase activity was determined in the presence of (mM): 5 ATP, 5 $MgSO_4$, 30 Tris/Mes, 50 KCl, and 2 μM gramicidin at pH 6.5 and 38°C. The inset shows a Dixon plot of these data used to determine the K_i for vanadate inhibition of the reconstituted ATPase (Dixon & Webb, 1958)

state equilibrium reached between the active influx of H^+ due to the activity of the H^+-ATPase and the passive efflux or leak of H^+ back out of the vesicle. Addition of the protonophore, FCCP, returned the fluorescence to the initial level. Ionophore-reversibility of fluorescence quenching is indicative of the formation of a pH gradient across the vesicle membrane rather than an artifact related to dye binding.

The enhancement of H^+ transport following reconstitution is shown in Fig. 5. These are a series of quench assays measured on an equal protein basis (60 μg protein per assay). Clearly, the quenching of acridine orange fluorescence was enhanced in the reconstituted vesicle relative to the plasma membrane vesicle. While this enhancement may have resulted from alterations in vesicle size, vesicle permeability, or internal buffering strength, when compared with the ATPase data it seems likely that the enhancement of fluorescence quenching is at least in part due to purification of the H^+-ATPase during reconstitution. The solubilized enzyme, still in the presence of DOC, showed no transport activity. Fluorescence quenching of both native and reconstituted vesicles was reversible by FCCP in the presence of the K^+ ionophore, valinomycin, which was needed for charge equilibration.

The ATPase activity of the reconstituted vesicles was highly sensitive to inhibition by vanadate. Figure 6 shows the concentration dependence of vanadate inhibition of both the native and reconstituted ATPase. The K_i for vanadate (Na_3VO_4) inhibition of the H^+-ATPase decreased from 9.5 to 2.4 μM following reconstitution (Fig. 6 inset). The shift in the K_i suggests that inhibition is interfered with in the native membrane fraction. The high sensitivity of the reconstituted ATPase to vanadate is similar to that reported for the *Neurospora* plasma membrane ATPase which has a K_i of 0.4 to 1.0 μM vanadate (Na_3VO_4), depending on ionic conditions (Bowman & Slayman, 1979). A shift in the vanadate inhibition curve was also observed for the *Neurospora* plasma membrane ATPase following purification (Bowman et al., 1981). The K_i values for the reconstituted ATPase from red beet ranged between 2.6 and 6.0 μM vanadate and were determined according to Dixon and Webb (1958).

H^+ transport in native plasma membrane vesicles has been presented in a previous report (Bennett et al., 1984). H^+ transport was insensitive to inhibition by 100 mM NO_3^- and 5 mg/ml oligomycin, confirming that these vesicles were not of tonoplast or mitochondrial origin. Vanadate inhibition of this transport was variable, ranging from 20 to 80% inhibition by 100 μM Na_3VO_4 when assayed in the presence of KNO_3. After reconstitution, sensitivity of H^+ transport to vanadate inhibition was enhanced in the reconstituted vesicles relative to the native plasma membrane vesicles in that inhibition was much greater using the same concentration of vanadate. Figure 7*A* shows that when reconstituted vesicles were incubated in the presence of KNO_3 or KCl, rapid quenching was observed upon the addition of ATP : Mg. Vanadate was added to a final concentration of 50 μM at the point indicated after

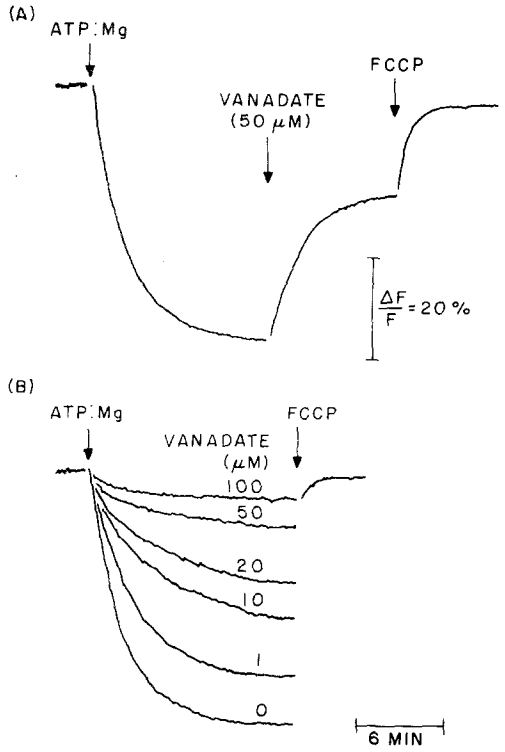

Fig. 7. Inhibition of H^+ transport by vanadate in reconstituted vesicles. Panel *A* shows the effect of vanadate (Na_3VO_4) when added to an equilibrated quench assay. Panel *B* shows the effect of vanadate (Na_3VO_4) when vesicles were preincubated with increasing concentrations of inhibitor from 0 to 100 μM. For both panels, ATP: Mg and FCCP were added to final concentrations of 5 mM and 0.5 μM, respectively. The reaction mix contained 10 mM Tris/Mes (pH 6.5), 25 mM KCl, 50 mM KNO_3, 5 μM acridine orange, and 35 μg membrane protein

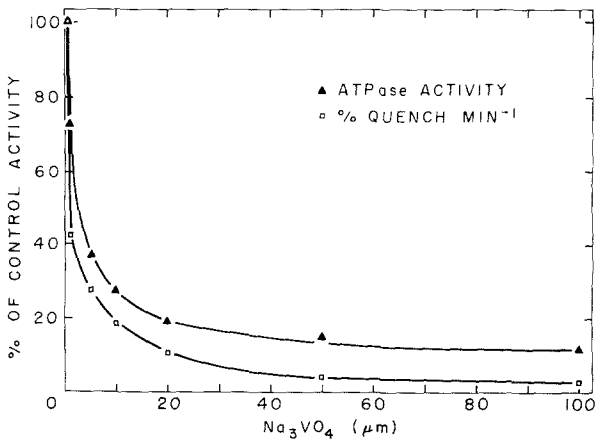

Fig. 8. Inhibition of ATPase activity and H^+ transport by vanadate. Data from Figs. 7 and 8 were replotted and normalized for the sake of direct comparison of the concentration dependence of vanadate inhibition

equilibrium between H^+ active influx and passive efflux had been reached. This resulted in an inhibition of the H^+-ATPase, revealing the passive leak of protons as a slow reversal of fluorescence quenching. In order to investigate the inhibition by vanadate of transport in the reconstituted vesicles more closely, a series of fluorescence quench curves were recorded for vesicles preincubated in the presence of increasing concentration of the inhibitor (Fig. 7*B*). Transport was progressively abolished, with half-maximal inhibition occurring at less than 10 μM vanadate.

The effects of vanadate on both ATPase and H^+ transport activity of the reconstituted enzyme are summarized in Fig. 8 where ATPase activity (from Fig. 6) and the initial rate of quench (from Fig. 7*B*) are normalized and plotted as a function of inhibitor concentration. Both functions are strongly inhibited by low concentrations of vanadate and have similar inhibition curves. The initial rate of quenching is proportional to the initial rate of H^+ transport (Bennett & Spanswick, 1983*a*; Wolosin & Forte, 1983). Thus both the initial rate of H^+ transport and ATPase activity appear to be equally sensitive to inhibition by vanadate, being inhibited half-maximally between 1 and 5 μM vanadate.

Cation Effects on Vanadate Inhibition

It has been shown for the Na^+/K^+-ATPase of animal cells and the H^+-ATPase of *Neurospora* that ionic conditions affect the extent of vanadate inhibition (Cantley, Cantley & Josephson, 1978; Bowman & Slayman, 1979). Cantley et al. (1978) have shown that the K_i for vanadate inhibition of the Na^+-ATPase is 200-fold higher (12 μM) than the K_i for vanadate inhibition of the Na^+/K^+-ATPase (50 nM). Bowman and Slayman (1979) reported that the K_i for vanadate inhibition of the *Neurospora* plasma membrane ATPase decreased from 1.0 μM in the absence of added cation to 0.64 μM in the presence of K^+, with Na^+ having no enhancement effect.

We examined the effect of K^+, Na^+ and combined K^+ and Na^+ on vanadate inhibition of the reconstituted H^+-ATPase of red beet plasma membrane (Fig. 9). Activity of the reconstituted ATPase in the absence of vanadate was stimulated 100% by K^+, 50% by Na^+ and 100% by combined K^+ and Na^+ (panel *A*). The K_i values for the four treatments were determined by plotting the data according to the method of Dixon and Webb (1958) as in panel *B*. The K_i for vanadate (V_2O_5) inhibition in the absence of added cations was 25.4 μM. The addition of K^+ lowered the K_i to 6.1 μM. Sodium ions had no effect on vanadate inhibition below 10 μM vanadate, yet inhibition of the ATPase was enhanced at higher

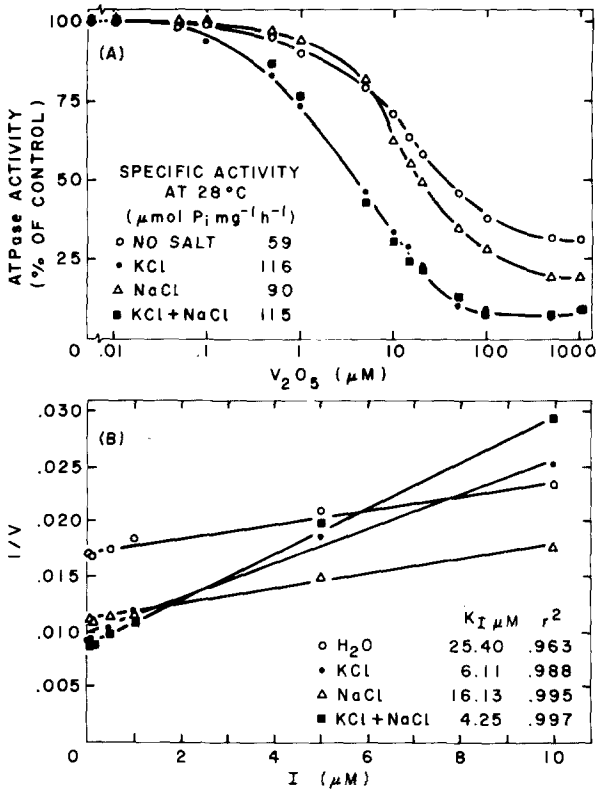

Fig. 9. Effects of cations on vanadate inhibition of reconstituted H^+-ATPase. Panel *A* shows the effect of increasing concentrations of vanadate (V_2O_5) on ATPase activity in the absence of added cations (○), or in the presence of K^+ (●), Na^+ (△) and combined K^+ and Na^+ (■). Panel *B* shows these data plotted according to the method of Dixon and Webb (1958). The inhibition curves were fit by regression analysis and the K_i values determined from these lines

concentrations of the inhibitor. The K_i in the presence of Na^+ was 16.1 μM.

Since Na^+ has been shown to stimulate red beet plasma membrane ATPase (Briskin & Poole, 1983*a*; O'Neill & Spanswick, 1984), a synergistic effect of K^+ and Na^+ on vanadate inhibition was possible. However, when K^+ and Na^+ were both present, the inhibition was no different than when only K^+ was present (panel *A*). The two curves are superimposable suggesting no enhancement of inhibition beyond the K^+-enhanced level. The K_i in the presence of K^+ and Na^+ was 4.25 μM, only slightly lower than that for K^+ alone. These results showing a lack of synergism agree with those of Briskin and Poole (1983*a*) who found no synergistic effect of K^+ and Na^+ on red beet plasma membrane ATPase activity, but not with those of Hansson and Kylin (1967) for sugar beet ATPase activity. The results suggest instead that Na^+ acts much like K^+ in being a stimulatory cation rather than as an ion directly involved in transport as with the Na^+/K^+-ATPase of animal cells.

The K^+ effect on vanadate inhibition is similar qualitatively to that reported for K^+ enhancement of vanadate inhibition of the Na^+/K^+-ATPase (Cantley et al., 1978) and the *Neurospora* ATPase (Bowman & Slayman, 1979). However, the Na^+ effect was more similar to that reported for the Na^+/K^+-ATPase (Cantley et al., 1978). The question arises concerning the role of Na^+ and K^+ in stimulating the plant plasma membrane ATPase. The lack of synergistic stimulation of the enzyme by K^+ and Na^+ in combination does not suggest a role for the Na^+ ion in direct transport by the ATPase as seen in the Na^+/K^+-ATPase. From examining the effects of monovalent cations on ATPase activity of both the native and reconstituted H^+-ATPase, it appears that Na^+ stimulates activity approximately 80% of the K^+ stimulated level (O'Neill & Spanswick, 1984). It seems likely that Na^+ can replace K^+ in stimulating the enzyme in a nonspecific manner. In this situation, Na^+ could replace K^+ and enhance vanadate inhibition by stimulating enzyme turnover. Briskin and Poole (1983*b*) have shown that KCl stimulates dephosphorylation of the red beet plasma membrane ATPase, and this would support the suggestion that Na^+ could act by replacing K^+ and thus enhance vanadate inhibition to a lesser extent.

In general, Dixon analysis of vanadate inhibition suggests that the reconstituted H^+-ATPase is a single enzyme with a monotonic inhibition curve and a single K_i. The K_i is altered by ionic conditions probably because cations, such as K^+ and Na^+, stimulate the enzyme's activity in a nonspecific manner, although there may also be an additional role for K^+ in direct transport by the enzyme.

Origin of the H^+-ATPase

In a previous report on red beet H^+-translocating ATPase (Bennett et al., 1984), we suggested a plasma membrane origin for the vanadate-sensitive, NO_3^- insensitive Na^+-ATPase based on marker enzyme and inhibition studies. It is currently thought that the plasma membrane is derived from Golgi membranes via transition vesicles, and thus it was of interest to examine the possibility that a portion of the H^+-ATPase activity of the 34/38% (wt/wt) fraction was of transition vesicle origin.

For this purpose, continuous sucrose gradients were examined for both vanadate-sensitive ATPase and UDPase activities. UDPase activity has been used as an enzymatic marker for Golgi membranes (Nagahashi & Kane, 1982). A representative gradi-

ent for red beet microsomal membranes is shown in Fig. 10. The peak of UDPase activity is separated clearly from the peak of vanadate-sensitive ATPase activity. UDPase activity reached a maximum between 1.11 and 1.12 g/cc (25.5 to 27.0% sucrose, wt/wt), and vanadate-sensitive ATPase activity reached a maximum between 1.15 and 1.16 g/cc (33.5 to 35% sucrose, wt/wt). The major peak of UDPase in red beet occurs at a density similar to that reported by Nagahashi and Kane (1982) for a corn root homogenate. Nitrate-sensitive ATPase activity peaked at a lower density of 1.09 g/cc (21.8% sucrose, wt/wt) as did the peak of H^+ transport (*not shown*).

Although the major peak of UDPase did not coincide with the vanadate-sensitive ATPase activity, there was a second smaller peak of UDPase activity occurring at about 1.16 g/cc that did coincide with this plasma membrane marker. From these continuous gradient results, clearly the major portion of Golgi membranes are not coincident with the plasma membrane H^+-ATPase activity. Yet this finding does not preclude the possibility that some of the H^+-ATPase activity may be associated with transition vesicles en route to the plasma membrane. The substrate specificity of the native plasma membrane and reconstituted ATPase described in the accompanying paper would suggest this possibility since UDP is second only to ATP as substrate for both the plasma membrane fraction and the reconstituted vesicles. In consideration of these results, we believe the vanadate-sensitive H^+-ATPase of the 34/38% (wt/wt) fraction represents the activity of a plasma membrane-localized ATPase; however, a portion of the vesicles may be transitional in nature and contain Golgi as well as plasma membrane characteristics.

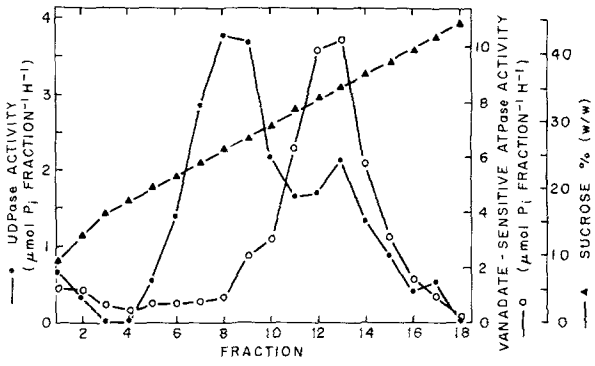

Fig. 10. Distribution of vanadate-sensitive ATPase and UDPase activities associated with red beet microsomal membranes on a continuous (12 to 45%, wt/wt) sucrose gradient. Vanadate-sensitive ATPase activity (○) was calculated as the difference in activity assayed in the presence and absence of 20 μM vanadate (V_2O_5). ATPase activity was assayed in the presence of 5 mM ATP/Tris, 5 mM M_gSO_4, 30 mM Tris/Mes, 50 mM KCl, and 2 μM gramicidin at pH 6.5 at 25°C. UDPase activity (●) was assayed in the presence of 0.1% Triton X-100 and as described in Materials and Methods. Sucrose concentration (%, wt/wt) is also shown (▲)

Conclusions

In this report, we have demonstrated the existence of a H^+-translocating ATPase associated with the plasma membrane of red beet. The H^+-ATPase was solubilized in active form and reconstituted into artificial lipid vesicles. This H^+-ATPase was characterized by a high sensitivity to vanadate. Vanadate is known to inhibit a certain class of ion-translocating ATPases that form a phosphorylated intermediate during the course of their reaction cycle (Macara, 1980), including the Na^+/K^+-ATPase of animal cell plasma membrane (Cantley et al., 1977), the H^+/K^+-ATPase of gastric mucosa, the Ca^{2+}-ATPase of sarcoplasmic reticulum (O'Neal, Rhoads & Racker, 1979), and the H^+-ATPase of fungal plasma membrane (Bowman & Slayman, 1979; Willsky, 1979). Inhibition by vanadate is specific for these enzymes only at low concentrations (less than 20 μM for half-inhibition of total activity).

Recently, Briskin and Poole (1983*a*) have characterized a plasma membrane fraction from red beet similar to that described in this report. Their plasma membrane preparation contained an ATPase that was inhibited 40% by 50 μM vanadate. The difference in inhibitor sensitivity may be related to differences in methods of membrane preparation and/or storage of tissue. Nonetheless, the two plasma membrane fractions have similar characteristics, and we believe we have isolated, solubilized, and reconstituted the same enzyme. Briskin and Poole (1983*b*) have also identified a phosphorylated intermediate associated with the red beet plasma membrane ATPase. The formation of a phosphorylated intermediate combined with the sensitivity to less than 20 μM vanadate for half-inhibition in our preparation permit the classification of this enzyme into the same group as the other major ion-translocating ATPases described above.

While other researchers have tentatively identified a plasma membrane H^+-ATPase (Sze & Churchill, 1981; Vara & Serrano, 1982), the beet plasma membrane system is clearly an improvement because we have been able to separate effectively the plasma membrane from the tonoplast and other membrane contaminants which may also be involved in H^+ transport. Furthermore, solubilization and reconstitution effect a three- to fourfold purification of the H^+-ATPase, resulting in an enhancement of vanadate-sensitive H^+ transport. Another

advantage is that beet storage tissue is an excellent source of both plasma membrane and tonoplast H^+-ATPase of considerably higher specific activity than found in many other plant tissues.

Finally, the H^+-ATPase activity described here most likely represents the activity of the electrogenic proton pump identified electrophysiologically by Poole (1974). Poole (1978) has further proposed that this pump catalyses H^+/K^+ exchange of variable stoichiometry. We have thus far examined only H^+ transport catalyzed by this enzyme; however, a future goal will be to examine the possible transport of K^+ by the reconstituted plasma membrane ATPase.

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