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Characterization of the Red Beet Plasma Membrane H^+-ATPase Reconstituted in a Planar Bilayer System[1,2]

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The transport activity of the red beet (*Beta vulgaris* L.) plasma membrane H^+-ATPase was examined following reconstitution into a planar bilayer membrane. Fusion of partially purified plasma membrane H^+-ATPase with the bilayer membrane was accomplished by perfusion of proteoliposomes against the bilayer under hypoosmotic conditions. Following incorporation into the bilayer, an ATP-dependent current was measured that demonstrated properties consistent with those of the plasma membrane H^+-ATPase. Current production was substrate specific for ATP, inhibited by orthovanadate, and insensitive to 200 nM erythrosin B but inhibited by 100 μM erythrosin B. When current production was measured as a function of Mg:ATP concentration, a simple Michaelis-Menten relationship was observed and a K_m of 0.62 mM was estimated. Current-voltage analysis of ATP-dependent current in the presence of 0.5 mM ATP, 20 mM ADP, 40 mM orthophosphate, and an opposing 2.5-unit ΔpH revealed a reversal potential of about −149 mV. Based on the free energy available from ATP hydrolysis, this reversal potential is consistent with an H^+/ATP stoichiometry of 1. This study demonstrates the usefulness of a planar bilayer system for investigation of energy coupling to H^+ transport by the plasma membrane H^+-ATPase.

The PM H^+-ATPase couples ATP hydrolysis to the extrusion of protons from the cytoplasm to the cell exterior (Briskin, 1990a, and refs. therein). This serves to establish a driving force for solute transport at the PM consisting of an acid-exterior pH gradient and interior-negative electrical potential difference (Briskin and Hanson, 1992; Chanson, 1993). Furthermore, the PM H^+-ATPase appears to play a significant role in a number of important plant physiological processes, including cell elongation (Rayle and Cleland, 1992), stomatal movements (Assmann, 1993), and cellular responses to a number of factors, including plant growth regulators, light, and fungal toxins (Briskin, 1990a; Palmgren, 1991; Briskin and Hanson, 1992; Vera-Estrella et al., 1994, and refs. therein).

The PM H^+-ATPase is a "P-type" transport ATPase that forms a covalent phosphorylated intermediate during the course of ATP hydrolysis (Briskin, 1990a; Briskin and Hanson, 1992). In the native membrane, the enzyme has a relatively simple dimeric structure consisting of two 100-kD catalytic subunits (Briskin and Hanson, 1992). Although the PM H^+-ATPase has been extensively studied in native membrane vesicle, detergent-solubilized, and reconstituted preparations (Briskin, 1990a; Briskin and Hanson, 1992, and refs. therein), little is known regarding the mechanism by which the exergonic reaction of ATP hydrolysis is coupled to the endergonic process of H^+ translocation.

To investigate the mechanism of energy coupling to H^+ translocation by the PM H^+-ATPase, both ATP hydrolytic and H^+-pumping activity must be analyzed under strictly defined conditions. In this respect, a planar bilayer system could prove extremely useful, since chemical conditions on both sides of the membrane as well as the electrical potential across the membrane can be precisely controlled (see Hanke and Schlue, 1993, for review). Although the planar bilayer system has been extensively used for examination of ion channels isolated from animal cells (Miller, 1986; Bridges and Benos, 1990; Hanke and Schlue, 1993, and refs. therein) and, more recently, plant cells (White and Testor, 1992, 1994, and refs. therein), this system has been used only in a few instances for the study of reconstituted primary transport ATPases, including the animal cell Na^+,K^+-ATPase (Jain et al., 1972), sarcoplasmic reticulum Ca^{2+}-ATPase (Nishie et al., 1990), and bacterial F_1F_0-ATPase (Hirata et al., 1986; Muneyuki et al., 1989). Recently, Ziegler et al. (1993) also reported functional reconstitution of the fungal PM H^+-ATPase in a planar bilayer membrane.

In this article, we describe reconstitution of the red beet (*Beta vulgaris* L.) PM H^+-ATPase in a planar bilayer and the initial characterization of its activity using this system.

MATERIALS AND METHODS

Plant Material

Red beets (*Beta vulgaris* L., var Detroit Dark Red) were purchased commercially. The leafy tops of the plants were removed and the storage tissue was placed in moist vermiculite and maintained at 4°C for at least 2 weeks to ensure uniformity in membrane isolation (Poole et al., 1984).

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Abbreviations: BTP, Bistris propane; PM, plasma membrane.

Preparation of Partially Purified Red Beet PM H^+-ATPase

PM fractions were prepared from red beet storage tissue as described by Gildensoph and Briskin (1990). PM vesicles were recovered from discontinuous Suc density gradients and collected by centrifugation at 100,000*g* for 1 h. The membrane fraction was extracted with 0.1% deoxycholate and the H^+-ATPase was then solubilized using 0.1% Zwittergent 3–14 and reconstituted into preformed liposomes as described by Singh et al. (1987). The liposomes were produced from soybean phospholipids (Asolectin) that had been further purified by solvent partitioning (Singh et al., 1987). Following elution in the void volume from a Sephadex G-200 column, the proteoliposomes were collected by centrifugation at 250,000*g* for 30 min in a Beckman TL-100 ultracentrifuge and suspended in 250 mM Suc, 2 mM BTP/Mes, pH 7.2, 10% glycerol, 2 mM DTT to a protein concentration of about 5 mg/mL. The final PM H^+-ATPase preparations typically had a specific activity of about 3.67 μmol min^{-1} mg^{-1} protein and could be frozen under liquid nitrogen and stored at −80°C without loss of activity for at least 3 months.

Production of Planar Bilayer Membranes

Planar bilayer membranes were produced across a 0.25-mm aperture separating two chambers. Soybean phospholipids were purified as described by Singh et al. (1987) and dissolved in *n*-decane at a concentration of about 25 mg/mL. The aperture was pretreated with the lipids in *n*-decane and air dried to form an "annulus" (Hanke and Schlue, 1993, and refs. therein). The bilayer chamber was then assembled and the lipids in *n*-decane were painted across the aperture using a fine glass rod. The thinning of the lipid to form a bilayer membrane was monitored by the measurement of membrane capacitance as described by Alverez (1986).

Fusion of H^+-ATPase-Containing Liposomes with the Bilayer

Fusion of the H^+-ATPase (in liposomes) with the planar bilayer was conducted in the presence of 50 mM Suc, 25 mM BTP/Mes, pH 6.5, 10 mM KCl, 2 mM $MgCl_2$, and 1 mM DTT (both chambers). Ten microliters of H^+-ATPase-containing liposomes (about 40 μg of protein) in 250 mM Suc, 1 mM BTP/Mes, pH 7.2, 1 mM DTT were slowly injected in one chamber adjacent to the bilayer (*cis* chamber) and mixing was applied for about 20 min using a magnetic stir bar. The extent of fusion with the planar bilayer was monitored by the decrease in membrane capacitance following injection of the H^+-ATPase preparation as described by Hirata et al. (1986). When membrane capacitance reached a minimum level (about 35–50 pF below the initial value), the solution on the *trans* chamber was transiently lowered below the aperture and then raised to its original level to promote liposome fusion with the bilayer (Hirata et al., 1986). Then the solution in the *cis* chamber was exchanged with fresh assay solution to remove residual, nonfused liposomes.

The number of H^+-ATPase molecules reconstituted into the planar bilayer (N_{ATPase}) was estimated from the measured level of ATP-dependent current based on the following equation:

$$N_{ATPase} = \text{Pump current/Faraday constant} \times 1/\text{specific activity} \times 1/\text{enzyme mol wt} \times \text{purity factor} \times 6.02 \times 10^{23} \quad (1)$$

where the pump current is expressed in Coulomb min^{-1} (i.e. 1 A =1 Coulomb s^{-1}), the purity factor represents the fractional purity of the preparation (i.e. fully pure = 1.0), and the Faraday constant is 96,500 cal mol^{-1}. It is also assumed that the stoichiometry of H^+ transport is 1 (Briskin and Reynolds-Niesman, 1991; see "Results and Discussion").

Measurement of ATP-Dependent Current

ATP-dependent current was typically measured in the presence of 50 mM Suc, 25 mm BTP/Mes, pH 6.5, 10 mm KCl, 3 mm $MgCl_2$, and 3 mM ATP (BTP salt, assay pH). The electric current was measured and voltage across the chambers was controlled using electronic equipment built by one of the authors (D.P.B.). The electrical potential of the *trans* chamber was held at virtual ground and command voltages were fed to the *cis* chamber via a similar electrode. The output from the I-V converter was amplified, conditioned using a Butterworth low-pass filter at 50 Hz, digitized, and then passed to a Macintosh computer. Data acquisition and signal analysis were conducted using a software package written by one of the authors (D.P.B.) in compiled QuickBASIC for Apple Macintosh.

Any variations in assay conditions are indicated in the figure legends.

Protein Determination

Protein was determined by the method of Bradford (1979) using BSA as a standard. The Bradford color reagent was filtered immediately prior to use.

RESULTS AND DISCUSSION

Fusion of proteoliposomes containing the partially purified red beet PM H^+-ATPase with a planar bilayer was accomplished by gentle perfusion against a planar bilayer membrane produced across a 0.25-mm aperture. The fusion of membrane vesicles (or proteoliposomes) with a planar bilayer is believed to take place in two stages, involving an initial vesicle adhesion to the *cis* side of the bilayer (i.e. the side to which the vesicles are added) followed by an actual fusion into the bilayer, where the luminal side of the vesicle membrane then faces the *trans* side (Akabas et al., 1984; Cohen and Niles, 1993). Adhesion of membrane vesicles to a planar bilayer is typically promoted by the presence of a divalent cation (Akabas et al., 1984; Hanke and Schlue, 1993) and the inclusion of 2 mM Mg^{2+} was found to be optimal for proteoliposomes containing the red beet PM H^+-ATPase and a bilayer produced from soybean phospholipids.

Although previous studies have found that the presence of an osmotic gradient across the bilayer (*cis* hyperosmotic relative to *trans*) enhanced adhesion and fusion (Cohen and Niles, 1993, and refs. therein), in our system this appeared to have only a minor impact on the extent of fusion with the bilayer (data not shown). On the other hand, optimal fusion with the bilayer was achieved when a 5-fold osmotic gradient was present across only the proteoliposome membrane, with the interior of the proteoliposome being hyperosmotic relative to the solution present in the *cis* chamber of the bilayer apparatus (see "Materials and Methods"). This ability of a vesicle or liposome osmotic gradient (rather than a bilayer osmotic gradient) to enhance fusion with the bilayer was also noted by Hanke and Schlue (1993). As assayed by the decrease in membrane capacitance (Hirata et al., 1986) or the magnitude of currents generated by the reconstituted H^+-ATPase, fusion appeared to be complete following 20 min of incubation with the bilayer.

When 3 mM Mg:ATP was added to the *cis* chamber of the bilayer apparatus, an electric current was generated that reached a steady-state level of about 15 to 20 pA (Fig. 1A).

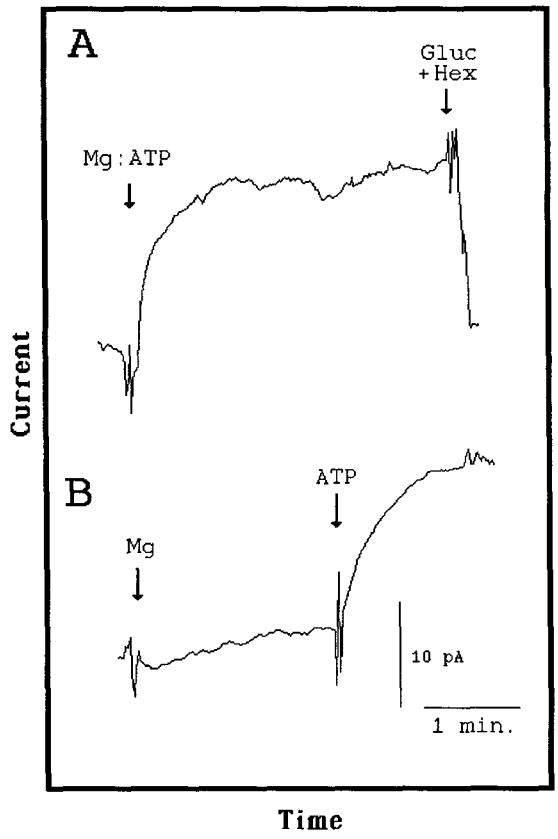

Figure 1. ATP-dependent current production by the red beet PM H^+-ATPase reconstituted in a planar bilayer. Current production was conducted in the presence of 50 mM Suc, 25 mM BTP/Mes, pH 6.5, and 10 mM KCl present on both sides of the bilayer membrane. A, The reaction was initiated by the addition of 3 mM Mg:ATP to the *cis* chamber, and 30 μL of a solution containing 1 M Glc (Gluc) and 60 units of hexokinase (Hex) were subsequently added as indicated. B, Three millimolar $MgCl_2$ and 3 mM ATP were added sequentially to the *cis* chamber as indicated.

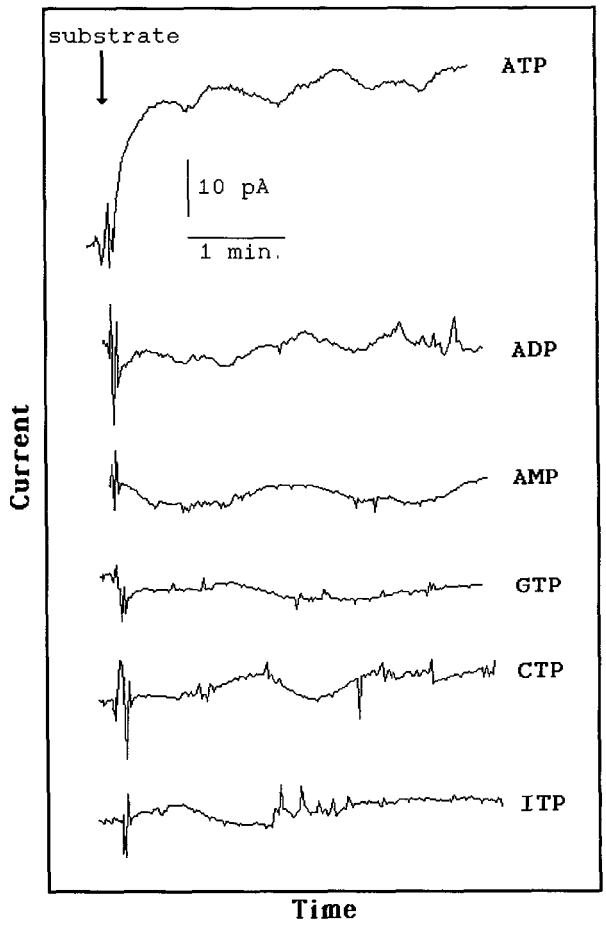

Figure 2. Substrate specificity for current production by the red beet PM H^+-ATPase reconstituted in a planar bilayer. Current generation was measured in the presence of 50 mM Suc, 25 mM BTP/Mes, pH 6.5, and 10 mM KCl present on both sides of the bilayer membrane. As indicated, 3 mM $MgCl_2$ and 3 mM substrate were injected into the *cis* chamber and the current was measured.

The sign of the electric current corresponded to a flow of positive charge from the *cis* chamber to the *trans* chamber. When ATP was removed by either perfusion of the *cis* chamber with an ATP-free transport buffer (data not shown) or the addition of Glc plus hexokinase (Fig. 1A), the electric current rapidly returned to the initial baseline level. This demonstrated that current production requires the presence of Mg:ATP. Further evidence for a specific ATP requirement comes from the observation that current production did not occur with the addition of Mg alone (Fig. 1B).

Current production was substrate specific for ATP, because the addition of other nucleoside phosphate compounds did not result in electrical current generation across the bilayer membrane (Fig. 2). Substrate specificity for ATP is a characteristic property of the PM H^+-ATPase, which is observed for both nucleoside phosphate hydrolysis and H^+ transport (Briskin, 1990a; Briskin and Hanson, 1992; Chanson, 1993, and refs. therein). On the other hand, the plant PM Ca^{2+}-ATPase can utilize GTP or ITP as a

substrate for hydrolysis and for driving Ca^{2+} transport (Briskin, 1990b; Williams et al., 1990; Chanson, 1993). Since current production was not observed in the presence of these substrates, it is unlikely that the PM Ca^{2+}-ATPase (as a possible contaminant) contributes to the observed electric current generated in the presence of ATP.

Current production in the reconstituted bilayer was partially inhibited by 10 μM Na_3VO_4 but fully inhibited by 100 μM Na_3VO_4 (Fig. 3). Although current production was insensitive to 200 nM erythrosin B, the addition of 100 μM erythrosin B resulted in a relatively rapid inhibition of activity (Fig. 4). These responses of ATP-dependent current production to Na_3VO_4 and erythrosin B are similar to what has been previously observed for the red beet PM H^+-ATPase (Giannini et al., 1987; Giannini and Briskin, 1987; Williams et al., 1990) and the enzyme examined in other plant species (Briskin, 1990a; Briskin and Hanson, 1992; Chanson, 1993, and refs. therein). Insensitivity to 200 nM erythrosin B, which fully inhibits the PM Ca^{2+}-ATPase (Briskin, 1990b; Williams et al., 1990), provides further evidence that the Ca^{2+}-ATPase does not contribute to the measured ATP-dependent current production.

When steady-state electric current production was examined as a function of ATP concentration, a simple Michaelis-Menten kinetics relationship was observed (Fig. 5). A re-plot of the data as a Hanes-Woolf plot revealed a linear relationship (r^2 = 0.932) and a K_m value for Mg:ATP of about 0.62 mM. This value is within the range of K_m values for Mg:ATP that have been previously observed for the plant PM H^+-ATPase (Briskin, 1990a; Briskin and Hanson, 1992, and refs. therein).

When taken together, these properties of the electrical current generated in the presence of Mg:ATP are consistent with this current being generated by the red beet PM H^+-ATPase after reconstitution into a planar bilayer. Based on the observed steady-state level of current production (18 pA), an estimate can be made for the number of H^+-ATPase molecules reconstituted into the bilayer (see "Materials and Methods"). Given the observed specific activity of the enzyme, a transport stoichiometry of 1 H^+ pumped per ATP hydrolyzed (Briskin and Reynolds-Niesman, 1991; see below), a molecular mass of 200 kD for reconstituted H^+-ATPase (Briskin and Reynolds-Niesman, 1989), and the assumption that the red beet H^+-ATPase preparation is about 50% pure on a protein basis, this flow of charge results in an estimate of about 4.6 million H^+-ATPase molecules being associated with the bilayer. From the specific activity alone it can be estimated that about 3.04×10^{-9} mg of protein were reconstituted into the bilayer. Since about 40 μg of enzyme preparation were injected into the *cis* chamber, this represents a reconstitution of about 7.6 millionths of a percent of the original preparation on a protein basis.

That a current of 18 pA represents the activity of about 4.6 million H^+-ATPase molecules clearly demonstrates the major difference in ion conductance between a primary transport pump such as the PM H^+-ATPase and an ion channel. Although ion channels only pass current during the frequent, brief periods when they are open (as opposed to the continuous operation of a pump), even a single, open channel can have current flow of this magnitude (Stein, 1986, and refs. therein). This difference in conductance level per protein is related to the fact that pumps mediate energy coupling and transport through relatively slow conformational change mechanisms while channels open to provide rapid passive ion movement through a transmembrane pore (Stein, 1986; Briskin and Hanson, 1992, and refs. therein).

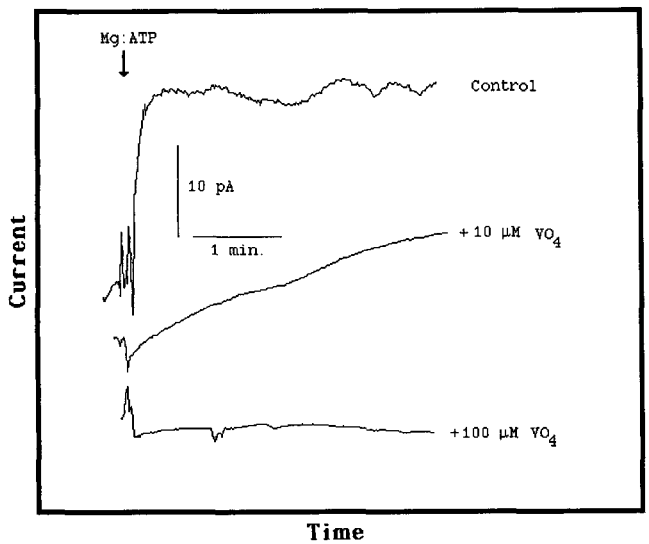

Figure 3. Effect of orthovanadate on ATP-dependent current production by the red beet PM H^+-ATPase reconstituted in a planar bilayer. Current generation was measured in the presence of 50 mM Suc, 25 mM BTP/Mes, pH 6.5, 10 mM KCl, and 3 mM Mg:ATP (1:1 concentration ratio, injected into the *cis* chamber), and the indicated concentration of Na_3VO_4 was also present in the *cis* chamber.

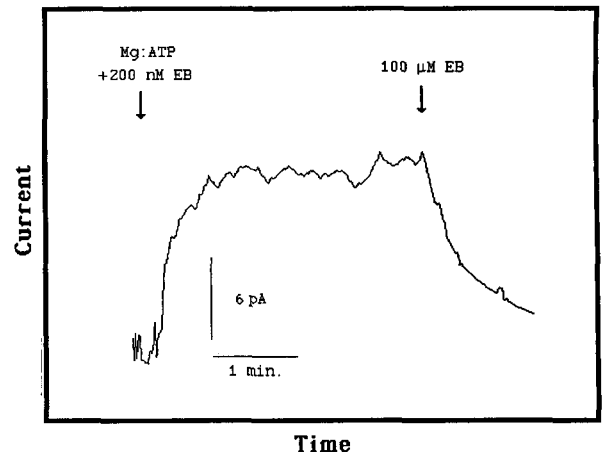

Figure 4. Effects of erythrosin B on ATP-dependent current production by the red beet PM H^+-ATPase reconstituted in a planar bilayer. Current production was measured in the presence of 50 mM Suc, 25 mM BTP/Mes, pH 6.5, 10 mM KCl, 3 mM Mg:ATP (1:1 concentration ratio, injected into the *cis* chamber), and 200 nM erythrosin B (EB) also present in the *cis* chamber. At the indicated time, an additional 100 μM erythrosin B was added to the *cis* chamber.

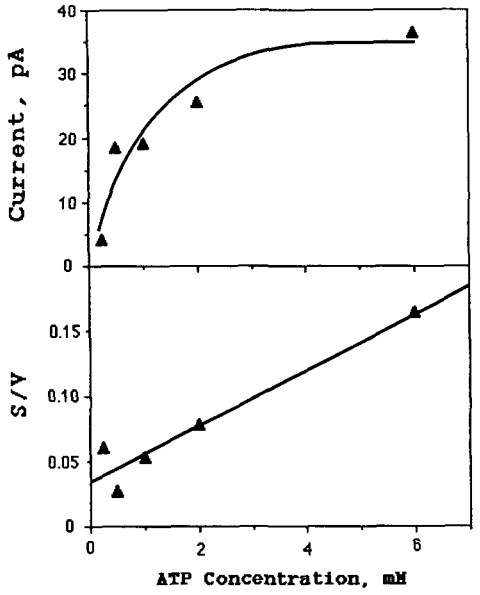

Figure 5. Effect of ATP concentration on current generation by the red beet PM H^+-ATPase reconstituted in a planar bilayer. Current generation was measured in the presence of 50 mM Suc, 25 mM BTP/Mes, pH 6.5, and 10 mM KCl, and the indicated concentration of Mg:ATP (1:1 concentration ratio) was injected into the *cis* chamber. Bottom, the data are re-plotted according to the Hanes-Woolf linear transformation of the Michaelis-Menten equation ($[S]/V = [1/V_{max}] \times [S] + K_m/V_{max}$). Analysis of the plot ($r^2 = 0.932$) yielded a K_m value of 0.62 mM.

Because the planar bilayer system allows control of both the chemical conditions and the electrical potential across the bilayer membrane, this system can be used to analyze the thermodynamic output of the PM H^+-ATPase under a given set of conditions. The basis for this technique is to determine the magnitude of an opposing $\Delta\mu H^+$ that can prevent measurable current generation by the PM H^+-ATPase. This value would represent the maximum output of the pump and would be related to the free energy of ATP hydrolysis by the following relationship:

$$\Delta G_{ATP\ Hydrolysis} = n \times \Delta\mu H^+ \quad (2)$$

where n refers to the stoichiometry of H^+ pumped per ATP hydrolyzed (Harold, 1986). The proton electrochemical gradient ($\Delta\mu H^+$) would, in turn, consist of both a pH gradient (ΔpH) and electrical potential difference ($\Delta\Psi$):

$$\Delta\mu H^+ = F \times (\Delta\Psi - 59\Delta pH) \quad (3)$$

where F represents the Faraday constant expressed as 23.06 kcal mol^{-1} V^{-1} (Harold, 1986). With our planar bilayer apparatus, the only limitation is that the maximum electrical potential that can be applied cannot exceed about ±185 mV, since this leads to a breakdown of the bilayer membrane (Hanke and Schlue, 1993, and data not shown).

As shown in Figure 6, the application of an opposing voltage across the bilayer leads to a decrease in ATP-dependent current production when measured at both pH 5.0 and 7.5 (on both sides of the bilayer membrane) and with [ATP] = 0.5 mM, [ADP] = 20 mM, and [Pi] = 40 mM. Nevertheless, the applied voltages within the range of the apparatus were insufficient to completely prevent net ATP-dependent current production even under reaction conditions in which the free energy of ATP hydrolysis was reduced relative to the "standard assay" (i.e. 3 mM Mg:ATP). On the other hand, if an opposing pH gradient (pH 7.5 on *cis* side; pH 5.0 on *trans* side) were also present across the bilayer membrane, ATP-dependent current production could be fully suppressed when the applied voltage (negative in *cis* chamber) reached about −147 mV.

Based on the magnitude of the opposing pH gradient across the bilayer (2.5 units) and the voltage observed to fully suppress ATP-dependent current production (−147 mV), the maximum thermodynamic output of the pump would correspond to a proton-motive force ($\Delta\mu H^+/F$) of about −295 mV. In terms of a proton electrochemical gradient, this would equal about −6.8 kcal mol^{-1}. From the relationship between $\Delta\mu H^+$ and $\Delta G_{ATP\ hydrolysis}$ at equilibrium (Eq. 2) and the estimated free energy available from ATP hydrolysis under these conditions (−7.36 kcal mol^{-1}), these data would also suggest a proton transport stoichiometry (n) of about 1.09 H^+ pumped per ATP hydrolyzed. This value is consistent with observed proton transport stoichiometries approaching 1, as estimated in studies conducted with the plant PM H^+-ATPases present in either native PM vesicles (Briskin and Reynolds-Niesman, 1991) or a reconstituted membrane system (Brauer et al., 1990).

In conclusion, the results of this study demonstrate the successful reconstitution of the red beet PM H^+-ATPase in a planar bilayer membrane. The current generated across

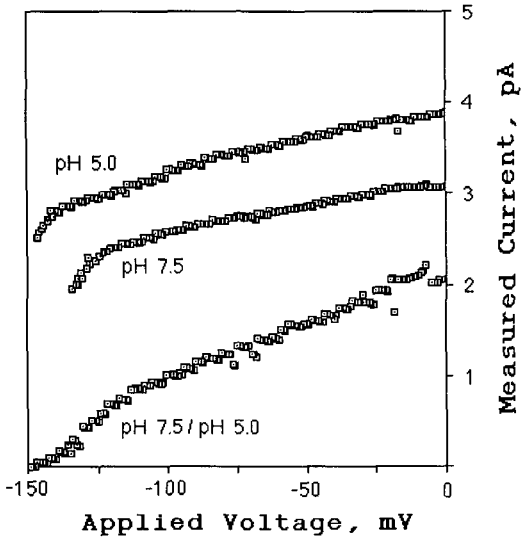

Figure 6. Effect of applied voltage on ATP-dependent current production by the red beet PM H^+-ATPase reconstituted in a planar bilayer. ATP-dependent current production was measured in the presence of 50 mM Suc, 25 mM BTP/Mes, pH 6.5, 2 mM $MgCl_2$, 10 mM KCl, 0.5 mM ATP (*cis* chamber), 20 mM ADP, and 40 mM Pi, and the assay pH was either 5.0 or 7.5 on both sides of the membrane or a 2.5-unit pH difference was present across the membrane (pH 5.0 *trans* side/pH 7.5 *cis* side). The voltage was clamped at the indicated values during ATP-dependent current production.

the bilayer demonstrates similar properties to those previously observed for ATP hydrolysis and H^+ pumping by this enzyme (Briskin, 1990a; Briskin and Hanson, 1992, and refs. therein) and this experimental system will be extremely useful for studies on the mechanism of energy coupling to H^+ transport by this enzyme. Although native membrane vesicles and reconstituted proteoliposomes have been useful in studying the mechanism of this enzyme (Briskin, 1990a; Briskin and Hanson, 1992, and refs. therein), these systems lack the rigorous control of transmembrane reaction/transport conditions. Likewise, although a solubilized enzyme preparation allows full access of sites on the enzyme to the enzyme substrate, transported solute, and possible effectors (e.g. K^+), transport studies are not possible with a solubilized preparation, because a membrane "compartment" is not present. Future studies will be conducted with the planar bilayer system to characterize the mechanism of H^+ transport by the PM H^+-ATPase, the role of K^+ in the reaction mechanism of the enzyme, and its regulation by various factors.

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