H⁺-ATPase Activity from Storage Tissue of Beta vulgaris

II. H⁺/ATP STOICHIOMETRY OF AN ANION-SENSITIVE H⁺-ATPase

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ABSTRACT

The H⁺/ATP stoichiometry was determined for an anion-sensitive H⁺-ATPase in membrane vesicles believed to be derived from tonoplast. Initial rates of proton influx were measured by monitoring the alkalization of a weakly buffered medium (pH 6.13) following the addition of ATP to a suspension of membrane vesicles of Beta vulgaris L. Initial rates of ATP hydrolysis were measured in an assay where ATP hydrolysis is coupled to NADH oxidation and monitored spectrophotometrically (A₂₅₄) or by monitoring the release of H⁺ from [γ-³²P]ATP. Inasmuch as this anion-sensitive H⁺-ATPase is strongly inhibited by NO₃⁻, initial rates of H⁺ influx and ATP hydrolysis were measured in the absence and presence of NO₃⁻ to account for ATPase activity not involved in H⁺ transport. The NO₃⁻-sensitive activities were calculated and used to estimate the ratio of H⁺ transported to ATP hydrolyzed. These measurements resulted in an estimate of the H⁺/ATP stoichiometry of 1.96 ± 0.14 suggesting that the actual stoichiometry is 2 H⁺ transported per ATP hydrolyzed. When compared with the reported values of the electrochemical potential gradient for H⁺ across the tonoplast measured in vivo, our result suggests that the H⁺-ATPase does not operate near equilibrium but is regulated by cellular factors other than energy supply.

Membranes isolated from red beet (Beta vulgaris L.) have been shown to contain two H⁺-translocating ATPases (5). In the accompanying paper (5), evidence was presented strongly supporting the proposal that the anion-sensitive H⁺-ATPase associated with low density membranes is of tonoplast origin. Furthermore, these low density membranes are tightly sealed vesicles as evidenced by the large ionophore stimulated activity of ATPase and by the ability to measure the generation of ATP:Mg-dependent pH gradients. The extremely high ionophore stimulated ATPase activity (>200%) in the low density membrane preparation suggested that a very high proportion, if not all, of the ATPase activity was present in sealed vesicles and coupled to H⁺ transport. It could, therefore, be a useful preparation in estimating the stoichiometry of coupling between H⁺ transport and ATP hydrolysis.

The determination of coupling stoichiometries for several ion-translocating ATPases has been a major goal in recent years (1, 12-14, 18, 22). A knowledge of the stoichiometry of coupling for Na⁺ and K⁺ transport by the Na⁺/K⁺-ATPase of animal cells is important in understanding the electrogenic properties of this ion-translocating ATPase (8). Similarly, a knowledge of the stoichiometry of coupling for H⁺ and K⁺ transport by the gastric H⁺/K⁺-ATPase is important in evaluating the energetic competence of this ATPase to sustain the large pH gradients observed in vivo (13, 14). The stoichiometry for H⁺ transport by the plasma membrane H⁺-ATPase of plant cells has also been of concern to plant cell electrophysiologists because this stoichiometry influences the maximal ion gradients sustainable across the plasma membrane (12, 18, 22). Estimates of the H⁺/ATP stoichiometry of the plasma membrane H⁺-ATPase of plant cells, based on electrophysiological evidence, indicate that 2 H⁺ are transported per ATP hydrolyzed whereas in Neurospora this stoichiometry is apparently 1 (22). Relatively little attention has been paid to the energetics of H⁺ transport at the tonoplast. However, recently the transport processes associated with this membrane have become accessible to biochemical characterization (5, 6, 10). In this report we look directly at the stoichiometry of coupling between H⁺ transport and ATP hydrolysis in a tonoplast membrane fraction.

A model illustrating the coupling of H⁺ transport to ATP hydrolysis by a H⁺-ATPase is shown in Figure 1. As indicated in equation 1 (Fig. 1), the maximal electrochemical potential gradient for H⁺ (δμH⁺) that can be formed by the H⁺-ATPase is a function of the free energy of hydrolysis of ATP (ΔG_ATP) and the stoichiometry of H⁺ transported per ATP hydrolyzed (n). If one assumes that the H⁺ pump is operating near equilibrium in vivo, then the H⁺/ATP stoichiometry (n) can be estimated from a measurement of δμH⁺ across the membrane in question and a knowledge of ΔG_ATP in the cellular environment (1). Using a similar, thermodynamic, approach with isolated membrane vesicles is complicated by the high conductance of the isolated vesicle membranes to H⁺ which prevents the development of maximal ion gradients in vitro (14).

In order to avoid problems associated with H⁺ leaks in membrane vesicles, a kinetic rather than thermodynamic approach has been favored in estimating H⁺/ATP stoichiometry of the gastric H⁺/K⁺-ATPase (13, 14). This kinetic method involves comparisons of the initial rates of proton influx with the initial rates of ATP hydrolysis. Under initial rate conditions, H⁺ gradients are small and so the initial rates of H⁺ transport should not be affected by H⁺ leaks. This latter approach has been used here to estimate the H⁺/ATP stoichiometry of the anion-sensitive H⁺-ATPase from red beets, believed to be of tonoplast origin (5).

MATERIALS AND METHODS

Membrane Preparation. Micromodal membranes from red beet (Beta vulgaris L.) storage tissue were prepared and low density membrane vesicles collected from a 16/26% (w/w) su-

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2 Present address: Department of Vegetable Crops, University of California, Davis, CA 95616.
3 Abbreviations: ΔG_ATP, free energy of ATP hydrolysis; ΔμH⁺, electrochemical potential gradient for H⁺; BTP, (1,3-bis[tris(hydroxymethyl)methylamino] propane; Δψ, membrane potential (mV)
crose discontinuous gradient as described in the accompanying paper (5). This membrane fraction was diluted in 2 mM Tris/Mes (pH 6.5), and was subsequently repelleted. The pellet was then resuspended in 250 mM sucrose, 0.5 mM MgSO₄, and 1 mM BTP/Mes (pH 6.13) at a concentration of 1.4 to 2.0 mg membrane protein/ml.

Proton Transport. Membrane vesicles (138 µl) were diluted in a stirred reaction cell at room temperature (approximately 23°C) with 862 µl of 250 mM sucrose, 0.5 mM MgSO₄, 1 mM BTP/Mes (pH 6.13), and either 58 mM KCl or 58 mM KNO₃. The pH of this vesicle suspension was then carefully adjusted to pH 6.13 with either 1 mM NaOH or HCl. Proton influx was initiated by the addition of 13 µl of 38 mM MgCl₂, 38 mM Na₂ATP with the pH adjusted to 6.13 with KOH. This gave final assay concentrations of 50 mM KCl or KNO₃, 1.0 mM Mg²⁺, 0.5 mM ATP, and approximately 225 µg membrane protein/ml.

Proton loss from the vesicle medium was measured with a semimicro combination glass pH electrode (Microelectrodes Inc., model MI-410) attached to a Keithley 604 electrometer and the amplifier output recorded with an MFE model 2125 chart recorder. Proton loss was calibrated after each measurement by the addition of 10 µl of either 1 mM NaOH or 1 mM HCl. The response to added H⁺ or OH⁻ for calibration indicated that the time required for mixing and electrode response was less than 1 s.

ATPase Assay. ATPase activity was determined by coupling ATP hydrolysis to NADH oxidation with P-enolpyruvate/pyruvate kinase and lactate dehydrogenase and monitoring NADH oxidation spectrophotometrically. Details of these coupling reactions are outlined by Auffret and Hanke (3). Membrane vesicles were diluted 5-fold with 250 mM sucrose, 0.5 mM MgSO₄, and 1 mM BTP/Mes (pH 6.13). A reaction mixture of 900 µl of 250 mM sucrose, 0.5 mM MgSO₄, 0.5 mM MgCl₂, 0.5 mM Na₂ATP, 5 units pyruvate kinase, 12 units lactate dehydrogenase, 0.42 mM P-enolpyruvate, 0.15 mM NADH, 1 mM BTP/Mes (pH 6.13), and either 50 mM KCl or KNO₃ was put into a 1.5 ml glass cuvette. The A at 340 nm was monitored with a Varian 634 spectrophotometer and recorded with an MFE model 2125 chart recorder. After recording a stable baseline A at 340 nm, the reaction was initiated by the addition of 100 µl of the diluted membrane vesicles (30 to 50 µg protein). Initial rates of NADH oxidation were calculated and equated with initial rates of ATP hydrolysis given that the coupling is 1 NADH oxidized/ATP hydrolyzed in this assay.

Alternatively, initial rates of ATPase activity were measured by monitoring the time course of 32P release from [γ-32P]ATP. Assays were set up exactly as for proton influx measurements (see previous section), except that the Na₂ATP, MgCl₂ added to initiate the reaction contained 1 µCi [γ-32P]ATP. At 30-s intervals, 100-µl aliquots of the reaction medium were removed and added to 1.9 ml 8% TCA. Inorganic phosphate was then complexed with molybdate by the addition of 500 µl 3.75% ammonium molybdate in 3 N H₂SO₄ and extracted in 2.5 ml butyl acetate (16). The time course of release of Pi was linear over 3 min, and the slope of the best fit linear regression fit to the data was used in the calculation of initial rates of [γ-32P]ATP hydrolysis.

Other Assays. Fluorescence quenching of 9-aminoacridine was assayed as described previously (5) in a medium identical to that used for proton influx measurements (see above). Excitation and emission wavelengths were 402 and 455 nm, respectively. Protein was determined by the method of Schaffner and Weissmann (17).

Source of Chemicals. ATP was obtained from Boehringer Mannheim as the disodium salt and used directly; [γ-32P]ATP was obtained from Amersham (specific radioactivity 3 Ci/mmole). Butyl acetate was from Fisher and all other chemicals were from Sigma.

RESULTS

The addition of Mg:ATP resulted in alkalization of the external medium of a membrane vesicle suspension (Fig. 2). The time course of alkalization is similar to that of fluorescence quenching of 9-aminoacidine and levels off after about 6 min (Fig. 2). The leveling off of fluorescence quenching has been previously attributed to active H⁺ influx and passive H⁺ efflux approaching equilibrium (6). This equilibrium of fluxes is also apparent in measurement of the alkalization of the vesicle
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external (Fig. 2). After the development of a pH gradient across the vesicle membrane, both the change in external medium pH and quenching of 9-aminoacridine fluorescence is completely reversed by addition of the channel-forming ionophore, gramicidin (Fig. 2). This complete reversal of the pH change indicated that the alkalinization resulted from H+ transport into the membrane vesicles and not from the production of H++OH- equivalents during ATP hydrolysis. At pH values as little as 0.2 unit below or above pH 6.13, production of H+ or OH- during ATP hydrolysis was sufficient to obliterate measurable pH changes resulting from H+ transport. A pH of 6.13 has been shown theoretically to be near the isoprotonic pH for ATP hydrolysis to ADP and P, under conditions utilized in this study (2). Although the presence of ADPase or adenylyl kinase activity can further influence the production of H+ or OH-, medium pH between 6.10 and 6.25 has been found to be suitable for measuring ATP-dependent H+ transport in submitochondrial particles (21) and gastric microsomal vesicles (13, 14).

The time course of ATP hydrolysis is also shown in Figure 2. The tracing is taken from a spectrophotometric recording of NADH oxidation (A,30) in the NADH-coupled ATPase assay (“Materials and Methods”). ATP hydrolysis is linear over the time span measured here (approximately 6 min) but increases sharply upon the addition of gramicidin. Based on measurements of the rates of ATP hydrolysis before the addition of gramicidin and on the initial rates of proton influx measured over time intervals before alkalinization begins to level off (typically over the interval between 6 and 30 s after ATP addition), the ratio of H+ transported per ATP hydrolyzed could be calculated.

A representative experiment is detailed in Table I where initial rates of H+ influx and ATP hydrolysis are measured in the presence of KCl or KNO3. With KCl present, rates of both H+ influx and ATP hydrolysis were higher than in the presence of KNO3, in agreement with our previous results indicating that NO3- is a selective inhibitor of this anion-sensitive H+-ATPase (5, 10). Contaminating phosphatase activity which did not contribute to H+ transport would result in an underestimate of the H+/ATP stoichiometry. We chose to base our calculations of the H+/ATP ratio on NO3- sensitive H+ transport and ATPase activities in order to avoid this source of error. The results shown in Table I are consistent with the presence of contaminating phosphatase activity which tends to lower the observed H+/ATP stoichiometry. The level of contamination varied from preparation to preparation resulting in calculated H+/ATP stoichiometries measured in the presence of KCl showing wide variations between 0.8 and 1.7 whereas the ΔNO3- H+/ATP stoichiometry was consistent from preparation to preparation as shown in Table II. The mean stoichiometry of H+/ATP coupling calculated from the values in Table II was 1.96 ± 0.14 (sd) and, if we assume that the true stoichiometry is an integer value, this suggests that the true H+/ATP stoichiometry is 2.

DISCUSSION

The approach used here to arrive at an H+/ATP stoichiometry of 2 for the red beet anion-sensitive H+-ATPase is best described as a kinetic approach since it has relied on measurements of initial rates of H+ influx and ATP hydrolysis. Errors in these measurements, such as H+ leaks, would tend to raise the H+/ATP stoichiometry to values above 2. Thus, the value reported here is conservative. A further and necessary test of this estimated stoichiometry is to determine if this value is energetically sufficient to account for the ΔH+ values measured in vivo. This amounts to a thermodynamic test of the value arrived at by this kinetic approach.

Referring to equation 1, we can see that the maximum Δψ+ sustainable by an H+-ATPase with an H+/ATP stoichiometry of 1 is ΔGATP. In order to evaluate this in measurable terms, we need an estimate for ΔGATP in the plant cytoplasmic environment, which can be arrived at by considering that:

\[ ΔG_{ATP} = AG^{\text{water}} + RT \ln \frac{[ADP][P]}{[ATP]} \]  

(2)

where AGwater is the standard free energy of ATP hydrolysis, R is the gas constant (e.g., 1.987 cal mol⁻¹ K⁻¹), and T is absolute temperature (296 K). The standard free energy of ATP hydrolysis (ΔGwater) at pH 7.5 and 1.0 mM Mg²⁺ is approximately −9.5 kcal mol⁻¹ (2). ADP/ATP ratios are relatively constant at 0.3 in plant cells (7, 9), cytoplasmic phosphate concentration has been estimated to be 1 to 5 mm (19), and ATP concentrations close to 1 mm (11). Using these values, a temperature of 23°C, and equation 2, we can calculate cytoplasmic ΔGATP to be in range between −10 and −11 kcal mol⁻¹.

The electrochemical potential gradient for H+ (Δψ+) in units of kcal mol⁻¹ is described by:

\[ Δψ = FΔψ - 2.303RT ΔpH \]  

(3)

where Δψ is the membrane potential in mv, F is the Faraday constant and ΔpH is the pH gradient across the membrane. Substituting equation 3 into equation 1 and rearranging we can arrive at an equation relating ΔGATP to measurable quantities of Δψ and ΔpH across a membrane:

\[ \frac{1}{n} ΔG_{ATP} ≥ Δψ - 2.303RT ΔpH. \]  

(4)

From equation 4, we can calculate the maximal pH gradients sustainable by an H+-ATPase operating at thermodynamic equilibrium over a range of possible membrane potentials (Fig. 3). The three lines in Figure 3 represent the maximum Δψ+ sustainable by a H+-ATPase with H+/ATP stoichiometries of either 1, 2, or 3 so that areas above each line are thermodynamically impossible, given their respective stoichiometries, and ΔGATP of

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Table II. Stoichiometry of NO3- Sensitive Proton Uptake to NO3- Sensitive ATP Hydrolysis

<table>
<thead>
<tr>
<th>Experiment</th>
<th>NO3- Sensitive H+ Influx</th>
<th>NO3- Sensitive ATP Hydrolysis</th>
<th>H+/ATP</th>
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</thead>
<tbody>
<tr>
<td>Experiment</td>
<td>µmol H+/mg-h</td>
<td>µmol P_/mg-h</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.35</td>
<td>3.38</td>
<td>1.88</td>
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<tr>
<td>2</td>
<td>7.26</td>
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<tr>
<td>3</td>
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<td>4</td>
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<td>1.99</td>
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<tr>
<td>5</td>
<td>10.95</td>
<td>4.95</td>
<td>2.21</td>
</tr>
<tr>
<td>6</td>
<td>7.47</td>
<td>3.89</td>
<td>1.92</td>
</tr>
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</table>

H+ influx was measured at pH 6.13 as described in "Materials and Methods." ATPase activity was assayed in experiments 1 to 4 with the NADH coupled assay and in experiments 5 and 6 by monitoring the release of 32P from [γ-32P]ATP.

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Table I. H+ Influx and ATPase Activity Measured in the Presence of Either KCl or KNO3

<table>
<thead>
<tr>
<th>Salt</th>
<th>H+ Influx</th>
<th>ATPase Activity</th>
<th>H+/ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol H+/mg-h</td>
<td>µmol P_/mg-h</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>13.86</td>
<td>9.83</td>
<td>1.41</td>
</tr>
<tr>
<td>KNO3</td>
<td>4.09</td>
<td>4.92</td>
<td>0.83</td>
</tr>
<tr>
<td>ΔNO3-</td>
<td>9.77</td>
<td>4.91</td>
<td>1.99</td>
</tr>
</tbody>
</table>

H+ influx and ATPase activity were assayed at pH 6.13 as described in "Materials and Methods."
Studies of the energetics of malate accumulation in the vacuole of a Crassulacean acid metabolism plant, Kalanchoe tubiflora, allowed an estimate of the H\(^+\)/ATP stoichiometry of a tonoplast H\(^+\)-ATPase responsible for malic acid accumulation in this plant (19). The results of this study were based strictly on a thermodynamic consideration of the free energy available from ATP hydrolysis and \(\Delta\mu_{H^+}\) across the tonoplast in vivo and resulted in an estimate of an H\(^+\)/ATP stoichiometry of 2. The close agreement in values based on work in vivo and in vitro is not necessarily expected since the tonoplast H\(^+\)-ATPase is most likely under kinetic regulation in vivo. The fact that the two values do agree suggests that in Crassulacean acid metabolism plants the tonoplast H\(^+\)-ATPase does indeed operate near thermodynamic equilibrium at the end of the dark period when malate accumulation in the vacuole is greatest (19).

**LITERATURE CITED**

15. Roberts JKM, PM Ray, N WADE-JARDETZKY 1980 Estimation of cytoplasmic and vacuolar pH in higher plant cells by \(^{31}P\)-NMR. Nature (Lond) 283: 870–872