Characterization of a K⁺- Stimulated Adenosine Triphosphatase Associated with the Plasma Membrane of Red Beet

DONALD P. BRISKIN AND RONALD J. POOLE
Department of Biology, McGill University, Montréal, Québec Canada H3A 1B1

ABSTRACT

A membrane fraction enriched with a magnesium-dependent, monovalent cation-stimulated ATPase was isolated from red beet (Beta vulgaris L.) storage roots by a combination of differential centrifugation, extraction with KI, and sucrose density gradient centrifugation. This fraction was distinct from endoplasmic reticulum, Golgi, mitochodrial, and possibly tonoplast membranes as determined from an analysis of marker enzymes. The ATPase activity associated with this fraction was further characterized and found to have a pH optimum of 6.5 in the presence of both Mg²⁺ and K⁺. The activity was substrate specific for ATP and had a temperature optimum near 40°C. Kinetics with Mg-ATP followed a simple Michaelis-Menten relationship. However the kinetics of K⁺-stimulation were complex and suggestive of negative cooperativity. When monovalent cations were present at 2.5 millimolarity, ATPase was stimulated in the sequence K⁺ > Rb⁺ > Na⁺ > Li⁺ but when the concentration was raised to 50 millimolarity, the sequence changed to K⁺ > Na⁺ > Rb⁺ > Li⁺. The activity was not synergistically stimulated by combinations of Na⁺ and K⁺. The enzyme was insensitive to NaN₃, oligomycin, ouabain, and sodium molybdate but sensitive to N,N'-dicyclohexylcarbodimide, diethylstilbestrol, and sodium vanadate. Based on the similarity of the properties of this ATPase activity and those from other well characterized plant tissues, it has been concluded that this membrane fraction is enriched with plasma membrane vesicles.

Plant storage tissue has provided a convenient system for the study of cellular ion transport. The use of storage tissue in ion flux studies has the advantage that transport can be considered for individual cells unaffected by long distance transport, growth, and photosynthesis (19). Ion transport in storage tissue has been well characterized (19 and references therein) and for red beet, ATP has been shown to be the energy source (18). Energy coupling for ion uptake into storage tissue may be mediated by a transport pump(s) present on the plasma membrane which may act in electrogenic proton efflux and/or coupled proton/potassium exchange (15).

Plasma membrane-enriched fractions have been isolated from numerous plant species and these have generally been shown to contain a Mg-dependent, monovalent cation-stimulated ATPase activity (11 and references therein). Whereas this enzyme has not been fully purified and its transport properties demonstrated in reconstituted membrane systems, much indirect evidence suggests that it may act in energy coupling to ion transport in higher plant cells (8, 11, 23).

We had some specific reasons for our interest in the plasma membrane ATPase of red beet. First, beet storage tissue is available in bulk and thus potentially suitable for enzyme purification and for phosphorylation studies. Secondly, we were interested to know to what extent the unusual transport features of beet (19)—its halophytic nature, propensity for accumulation of sodium, and the large effect of pH on cation accumulation—might be related to the properties of the plasma membrane ATPase. While the ATPase activity in crude membrane fractions from this species (sugar beet) has been analyzed (6), the properties of this activity in purified plasma membrane fractions have not been characterized.

In this paper, cell fractionation studies with red beet storage root are presented. A membrane fraction enriched with Mg-dependent, monovalent cation-stimulated ATPase activity distinct from ER, Golgi, and mitochondrial enzyme markers was prepared. The properties of the ATPase activity were similar to those reported for corn (12) and oat (8) root plasma membrane fractions.

MATERIALS AND METHODS

Plant Material. Red beet (Beta vulgaris L.) storage roots were purchased commercially. The tops of the plants were removed and the storage roots were stored in moist vermiculite at 5°C until use.

Isolation of Microsomal Membrane Fractions. Beet roots were rinsed with distilled H₂O and then cut into sections. All subsequent steps were carried out at 2 to 4°C. The storage tissue was homogenized with a Braun juice extractor in a medium containing 250 mM sucrose, 3 mM EDTA, 0.5% (w/v) PVP-40, 70 mM Tris-HCl, pH 8, and 4 mM DTE.² One ml of homogenizing medium was used per gram of tissue. The homogenate was filtered through four layers of cheesecloth and centrifuged at 13,000g (10,500 rpm) for 15 min in a Sorvall SS-34 rotor. The pellets were discarded and the supernatant was centrifuged at 80,000g (32,500 rpm) for 30 min in a Beckman Ti 70 rotor to obtain a microsomal pellet.

Treatment of the Microsomal Pellet with KI. The microsomal pellet was suspended in 250 mM sucrose, 1 mM Tris-Mes, pH 7.2, 1 mM DTE (suspension buffer) and the protein concentration was adjusted to about 1 mg/ml. An equal volume of suspension buffer containing twice the desired concentration of KI was added slowly with gentle mixing. The membranes were incubated on ice for 20 min and then centrifuged at 80,000g (32,500 rpm) for 30 min in a Beckman Ti 70 rotor. The supernatant was discarded and the pellet was used for further study.

Sucrose Density Gradient Centrifugation. The KI-extracted pellet was suspended in 0.5 ml suspension buffer with a glass-Teflon Dounce-type homogenizer and layered on discontinuous sucrose density gradients (Figure 1). All sucrose solutions were buffered with 1 mM Tris-Mes, pH 7.2, and contained 1 mM DTE. The gradients were centrifuged at 115,000g (26,000 rpm) for 2 h

² Abbreviations: DTE, dithioerythritol; DCCD, N,N'-dicyclohexylcarbodimide; DES, diethylstilbestrol; PNP, p-nitrophenyl phosphate.
PLASMA MEMBRANE ATPase OF RED BEET

Sample Overlay

Fig. 1. Gradients used to prepare membrane fractions from red beet storage tissue.

in a Beckman SW 41 Ti rotor. Gradient interface bands were removed with a Pasteur pipette.

Enzyme Assays. Phosphate hydrolyzing activity was measured in a 1.0-ml reaction volume containing 3 mM substrate, 3 mM MgSO₄, and 30 mM Tris-Mes (titrated from 0.3 M stocks to desired pH). For the standard ATPase assay, the pH was 6.5 and, when KCl was present, its final concentration was 50 mM. The reaction was carried out for 30 min at 38°C and the released Pi was determined by the method of Dupont et al. (4). Potassium-stimulated ATPase activity represented the difference between activity measured in the presence and absence of 50 mM KCl. Any variations on the assay conditions are indicated in “Results.” ATP was purchased from Boehringer and converted to the Tris salt by cation exchange on Dowex 50 resin. All other substrates were purchased from Sigma and were used as sodium salts.

Latent IDPase was measured as described by Ray et al. (21). Membrane samples were assayed upon isolation and after 6 d of storage at 2 to 4°C. Latent IDPase activity was considered as the difference in activity following storage (20).

NADPH Cyt c reductase and Cyt c oxidase were measured spectrophotometrically (13) at room temperature (22°C).

Protein Assay. Protein was determined by the method of Peterson (17) following a TCA precipitation to eliminate interference by DTE.

RESULTS

Treatment of the Microsomal Membranes with KI. Initial attempts to isolate plasma membrane fractions from red beet storage root using K⁺-stimulated ATPase as a marker were confounded by the presence of substantial levels of phosphatase activity. In this respect, membrane fractions isolated from red beet may be similar to those from barley roots where a large component of nonspecific phosphatase was present in addition to ATP-specific hydrolytic activity (16). It was found that treatment of the microsomal membranes with the cationic salt KI tended to reduce nonspecific phosphatase activity (measured by hydrolysis of p-nitrophenyl phosphate) relative to ATP hydrolytic activity (Fig. 2). Maximal enrichment of ATP hydrolytic activity relative to PNP hydrolytic activity occurred when the membranes were treated with 0.25 M KI. The observation that a portion of the phosphatase activity can be removed by KI would suggest that this activity might be localized on membrane peripheral proteins (25). Higher concentrations of KI tended to reduce ATPase activity while the residual PNPane activity appeared to be less sensitive to this treatment. Therefore, extraction of the microsomal membranes with 0.25 M KI was included as a step in the membrane isolation. This treatment resulted in a loss of 20 to 25% of the protein in the microsomal fraction to the high speed supernatant.

Centrifugation of the KI-Extracted Membranes on Sucrose Density Gradients. Microsomal membranes which were extracted with 0.25 M KI were then layered onto a discontinuous sucrose gradient (Fig. 1, Gradient 1). Following centrifugation at 115,000g for 2 h, the gradient fractions were removed and assayed for various marker enzymes (Table I). Gradient fraction C (25/30 sucrose interface) appeared to be enriched in pH 6.5 ATPase and also K⁺-stimulated ATPase, indicating possible enrichment with plasma membrane vesicles. ATP hydrolytic activity at pH 8.5 was assayed for the fractions and was found both at low and high sucrose density. The high density activity coincided with the distribution of Cyt c oxidase, a marker for mitochondrial membranes and may represent the activity of mitochondrial ATPase (13). This would be consistent with the observation that this activity was strongly inhibited by 1 mM NaN₃ (data not shown). The low density activity occurred in highly pigmented membrane vesicles that coincided with the density of ER as measured with NADPH-Cyt c reductase activity (20). Work currently in progress indicates that this activity is similar to that observed to be present with isolated vacuole fractions of red beet (9) and thus may represent an enzyme localized on the tonoplast membrane. Golgi membranes as determined by measuring latent IDPase activity showed a peak distribution at 30 to 35% sucrose.

The ATPase activity associated with gradient fraction C was further characterized so that its properties could be compared with the reported properties of plasma membrane ATPase from other plant tissues, notably the preparations isolated from corn (12) and oat (10, 11) roots. With these preparations, the identity of plasma membrane has been determined independently by the periodic acid–chromic acid–phosphotungstic acid–staining technique and K⁺-stimulated ATPase activity correlated well with the distribution of plasma membrane (8, 10, 11). Similarities between the properties of the ATPase activities would provide strong but not absolute evidence for the validity of using K⁺-stimulated ATPase as a marker enzyme for plasma membrane in red beet storage tissue. The membranes were isolated using the preparative gradient shown in Figure 1 (Gradient 2).

General Characteristics of the ATPase Activity. The ATPase activity associated with gradient-purified membranes of red beet displayed a pH optimum of 7.5 when assayed in the presence of 3 mM MgSO₄ (Fig. 3). When 50 mM KCl was included in the assay, the pH optimum declined to 6.5. The pH optimum of 6.5 in the presence of Mg⁺⁺ and K⁺ shows that it is not affected when the ATPase of 50 mM KCl is similar to what is observed for the plasma membrane ATPase of oat and corn roots (8, 12). This acidic pH optimum in the presence of Mg⁺⁺ and K⁺ however differs from that reported for an ATPase activity associated with the plasma membrane of cauliflower (14).

The temperature optimum for the ATPase was at 38°C (Fig. 4). The temperature optimum for the plasma membrane ATPase of oat and corn roots was also near 40°C (8, 12). Unlike the activity associated with corn root plasma membranes, substantial K⁺-stimulation was observed at lower temperatures.

The ATPase activity demonstrated a clear preference for ATP as a substrate (Table II). All components of the activity were substrate specific for ATP.

The activity displayed simple Michaelis-Menten kinetics when assayed at various concentrations of Mg:ATP (added as the 1:1 concentration ratio) in the presence or absence of 50 mM KCl (Fig. 5). The major effect of the addition of KCl was to increase the Kₘ for Mg:ATP. The Vₘₐₓ increased from 19.29 to 33.48 μmol Pi/mg h whereas the Kₘ decreased from 0.81 to 0.52 mM with the KCl addition. The Kₘ values for Mg:ATP were within the range of values reported for corn (12) and oat (8) root plasma membrane ATPase.

Effects of Various Ions. The beet root ATPase activity required the presence of a divalent cation for activity (Table III). The greatest activity was observed with Mg⁺⁺ whereas other divalent cations showed lower effects. Stimulation of the enzyme by KCl

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was clearly dependent on the presence of a divalent cation with the exception of Fe²⁺, where K⁺ stimulation was absent. The enhancement of the basal activity by Mg²⁺ was maximal when ATP and Mg²⁺ were present in the 1:1 concentration ratio (data not shown). When the enzyme was assayed in the presence of Mg²⁺ or Mg²⁺ and K⁺, Ca²⁺ acted as an inhibitor. These observations are similar to what is found for the plasma membrane ATPase from other plant tissues and are consistent with Mg:ATP being the true substrate for the enzyme (8, 10). Calcium ion inhibits the activity by interfering with the formation of the Mg:ATP complex (12).

The activity of the beet root ATPase when measured in the presence of Mg²⁺ was stimulated by various monovalent cations. However K⁺ was the most effective (Table IV). The monovalent ion specificity was less marked than in corn (12) or oats (24) and was less pronounced at a salt concentration of 50 mM than at 2.5 mM.

Early studies by Hansson and Kylin (6) with crude membrane fractions from sugar beets indicated that ATPase in this preparation was synergistically stimulated by combinations of Na⁺ and K⁺. Thus, this enzyme showed a response somewhat like the animal cell (Na⁺, K⁺)-ATPase (7). In this study with gradient-purified membranes from the same species, these results could not be confirmed (Fig. 6). The membrane-bound ATPase of red beet storage tissue showed a similar response to combinations of Na⁺ and K⁺ as the plasma membrane ATPase of oat and corn roots (8, 10, 12).

The kinetics of K⁺ stimulation of ATPase for the red beet membranes did not fit the Michaelis-Menten relationship as
shown by the absence of a linear relationship when the data were analyzed with an Eadie-Hofstee plot (Fig. 7). Instead, complex kinetics consistent with negative cooperativity were observed. Complex kinetics of this type have been previously observed for stimulation of plant plasma membrane ATPase and ion uptake into plant tissue (8, 10, 12).

**Effects of Inhibitors.** The beet root ATPase activity was assayed in the presence of inhibitors of various phosphohydrolases (Table V). The enzyme was insensitive to the mitochondrial ATPase inhibitors oligomycin and azide and also insensitive to ouabain, an inhibitor of the animal cell (Na+, K+)-ATPase (7). Molybdate, an inhibitor of acid phosphatase (3) was only slightly effective in inhibiting the activity. The activity, however, was sensitive to DCCD, DES, and vanadate. These agents have been shown to be inhibitors of plasma membrane ATPase in plants (1, 4, 8, 10).

**DISCUSSION**

In this study, a method is presented for the isolation of a membrane fraction enriched in a Mg-dependent, monovalent cation-stimulated ATPase from red beet storage tissue. This membrane fraction is distinct from ER, Golgi, mitochondrial, and possibly tonoplast membranes. When the properties of the ATPase were investigated, they were found to be very similar to those reported for the plasma membrane ATPase of oat and corn roots (8, 12). The preparations have a similar pH optimum and temperature optimum and are substrate specific for ATP. Substrate specificity for ATP is a general property of the plasma membrane ATPase of plant (8, 10, 11) and fungal (5) cells. It is also characteristic of vanadate-sensitive electrogenic transport activity in plant membrane vesicles (23), presumably originating from the plasma membrane. The kinetics with Mg:ATP and the response of the kinetics to the addition of KCl for the beet root ATPase are identical to what is observed for the plasma membrane ATPase of corn and oat roots (8, 10). For the beet root ATPase as well as the preparation from oat and corn roots, the true substrate for the

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**Table II. Activity of the Red Beet Plasma Membrane Fraction with Various Substrates**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( -K^+ )</th>
<th>( +K^+ )</th>
<th>( K^+ ) stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>20.1</td>
<td>45.8</td>
<td>25.7</td>
</tr>
<tr>
<td>ADP</td>
<td>4.1</td>
<td>5.6</td>
<td>1.5</td>
</tr>
<tr>
<td>AMP</td>
<td>1.3</td>
<td>1.9</td>
<td>0.6</td>
</tr>
<tr>
<td>GTP</td>
<td>3.1</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>UTP</td>
<td>0</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>ITP</td>
<td>4.5</td>
<td>6.0</td>
<td>1.5</td>
</tr>
<tr>
<td>PNP</td>
<td>5.0</td>
<td>6.6</td>
<td>1.6</td>
</tr>
</tbody>
</table>

**Table III. Effect of Various Divalent Cations on the ATPase Activity Associated with Red Beet Plasma Membranes**

<table>
<thead>
<tr>
<th>Divalent Cation</th>
<th>( -K^+ )</th>
<th>( +K^+ )</th>
<th>( K^+ ) stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.9</td>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>22.8</td>
<td>40.5</td>
<td>17.7</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>7.7</td>
<td>14.3</td>
<td>6.6</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>10.2</td>
<td>20.7</td>
<td>10.5</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>7.1</td>
<td>6.4</td>
<td>0</td>
</tr>
<tr>
<td>CaSO₄</td>
<td>1.4</td>
<td>2.0</td>
<td>0.6</td>
</tr>
<tr>
<td>MgSO₄ + 1.5 mM CaSO₄</td>
<td>17.1 (25%)</td>
<td>27.2 (33%)</td>
<td>10.1 (43%)</td>
</tr>
</tbody>
</table>

* Divalent cations were present at 3 mM assay concentration.

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**Fig. 4.** Effect of assay temperature on ATPase activity associated with gradient-purified membranes from red beet.

**Fig. 5.** Top, ATPase activity as a function of Mg:ATP concentration for gradient-purified membranes from red beet. Bottom, data plotted according to the linear transformation of the Michaelis-Menten equation.

\[
\frac{S}{V} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}} S
\]

Kinetic constants, determined by regression analysis, were \( K_m = 0.81 \) mM, \( V_{max} = 19.29 \) \( \mu \)mol Pi/mg-h in the absence of KCl and \( K_m = 0.52 \) mM, \( V_{max} = 33.48 \) \( \mu \)mol Pi/mg-h in the presence of KCl.
enzyme is most likely the Mg:ATP complex (10).

The relative effectiveness of monovalent cations (2.5 mM concentration) in stimulating ATPase in red beet was similar to that shown for oat and corn roots (10), but differences between the cations were less pronounced. As in corn (12) and unlike oats (24), the cation specificity decreased at higher salt concentrations. Although stimulation of the best ATPase by Na+ was relatively greater than that seen in earlier preparations of plasma membrane, it is not clear whether this is related to the preferential Na+ uptake seen under certain conditions in this halophyte. The interaction between Na+ and K+ uptake in beet cannot be explained by simple competition (19). Sze and Hodges (24) also conclude that in oats there is no close relationship between Na+ transport and Na+ stimulation of the ATPase.

The lack of synergistic, or even additive, stimulation by Na+ + K+ shows that this enzyme is neither a (Na+, K+)-ATPase nor a mixture of (Na+)-ATPase and (K+)-ATPase. The marked sensitivity of cation uptake to external pH observed in beet tissue (19) is not reflected in the pH response of the ATPase, which, as mentioned above, is very similar to that observed in oats and corn. The beet root enzyme showed complex negative cooperative kinetics for K+ stimulation and sensitivity to inhibitors of plasma membrane ATPase. Based on the major similarities between the ATPase activity present in beet root membranes and the plasma membrane fractions of corn and oat roots, we have concluded that pH 6.5 K+-stimulated ATPase most likely represents a valid marker for the plasma membrane of red beet and the membrane fraction isolated with plasma membrane. This conclusion is supported by the low activities of markers for other cellular components in this fraction.

Given that this preparation is enriched in plasma membrane, its density of 1.10 to 1.12 g/cc in sucrose gradients is much lower than the values generally shown for plasma membrane (8, 11, 20). In addition, the plasma membrane appears to be less dense than the Golgi membranes, contrary to what is generally observed (20). Whether this represents an unusual property of storage tissue or an effect of the treatment with KI (i.e., removing peripheral proteins) remains unclear at the present time. In a recent study by Robinson et al. (22) where membrane fractions were isolated from carrots, plasma membrane, marked by glucan synthetase II, showed a density similar to that reported for red beet in this study. With the isolation of plasma membrane from red beet storage tissue, the mechanism of energy coupling to ion transport can be investigated in a system well characterized as to the energy source for transport (18) and general properties related to transport (i.e., membrane potential, ion flux properties) (15, 18, 19, and reference therein). Work currently in progress suggests that energy coupling may occur with the formation of an ATPase phosphoenzyme intermediate similar to that observed for the transport ATPases of animal cells (7), fungal cells (5), and recently the plasma membrane ATPase of corn roots (2).

**Table IV. Effect of Monovalent Cations on the Plasma Membrane ATPase of Red Beet**

<table>
<thead>
<tr>
<th>Cation</th>
<th>2.5 mm</th>
<th>50 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>44.9 (100)</td>
<td>76.3 (100)</td>
</tr>
<tr>
<td>RbCl</td>
<td>42.7 (95.1)</td>
<td>75.3 (98.7)</td>
</tr>
<tr>
<td>NaCl</td>
<td>38.8 (86.5)</td>
<td>75.9 (99.4)</td>
</tr>
<tr>
<td>LiCl</td>
<td>28.7 (64.0)</td>
<td>68.1 (89.3)</td>
</tr>
</tbody>
</table>

* Values in parentheses indicate ATPase activity relative to that in the presence of KCl.

**Fig. 6.** Effect of various KCl and NaCl concentrations on the beet root membrane ATPase activity. ATPase was assayed as described in "Materials and Methods" in the presence of varying concentrations of KCl and NaCl. The total concentration of monovalent cations was maintained at 50 mM. The activity in the presence of 3 mM MgSO₄ was 31.5 μmol Pi/mg-h.

**Fig. 7.** Effect of increasing concentrations of KCl on the ATPase activity of red beet membranes. ATPase activity was assayed as described in "Materials and Methods" in the presence of increasing concentrations of KCl (0.1–50 mM). The data are presented as an Edel-Hofstee plot.

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