Characterization of Anion Effects on the Nitrate-Sensitive ATP-Dependent Proton Pumping Activity of Soybean (Glycine max L.) Seedling Root Microsomes

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ABSTRACT

The ATP-dependent proton-pumping activity of soybean (Glycine max L.) root microsomes is predominantly nitrate sensitive and presumably derived from the tonoplast. We used microsomes to characterize anion effects on proton pumping of the tonoplast vesicles using two distinctly different techniques. Preincubation of the vesicles with nitrate caused inhibition of proton pumping and ATPase activity, with similar concentration dependence. Fluoride, which preferentially inhibits the plasma membrane ATPase, inhibited ATPase activity strongly at concentrations which did not affect proton pumping activity.

Addition of potassium salts, after a steady-state pH gradient is established in the absence of such salts, caused an increased pH gradient which was due to alleviation of $\Delta \psi$ and subsequent increased influx of $H^+$ into these vesicles. This anion-induced increase in the pH gradient could be used as a measure of the relative union permeabilities, which were of the order $Br^- = NO_3^- > Cl^- > SO_4^{2-}$. Phosphate and fluoride caused no increase in the pH gradient. Since the concentration dependence of KCl- and KNO3-induced quenching exhibited a saturable component, and since $H^+$ uptake was increased by only certain anions, the data suggest that there may be a relatively specific anion channel associated with tonoplast-derived vesicles.

ATP-dependent $H^+$-pumping activities of a variety of plant tissues have been demonstrated and characterized (8, 10, 16, 20, 22, 24, 25). Two separate activities have been identified on the basis of the differential effects of the inhibitors nitrate and vanadate, and localization on density gradients (3, 6, 13). Nitrate-inhibited activity is located at low densities on sucrose density gradients and probably originates from the tonoplast (3, 7, 14). Vanadate-inhibited activity is located at high densities on gradients and probably originates from the plasma membrane (3, 6, 13).

The tonoplast-derived $H^+$-pumping activity is selectively stimulated by chloride (8, 10, 16, 22). Hager and Helmle (10) suggested that a chloride channel existed based on the inhibition of $H^+$ pumping by the anion channel blocker DIDS, and that chloride also specifically stimulates the $H^+$ pumping ATPase itself. Mettler et al. (16) showed that DIDS partially inhibited ATP-dependent methylene and $Cl^-$ uptake by tonoplast vesicles. Bennett and Spanswick (2) presented kinetic evidence in support of the presence of a $Cl^-$ channel, and also showed that DIDS selectively inhibits a saturable component of KCl-dependent $H^+$ pumping.

In soybean microsomes, nitrate-sensitive $H^+$-pumping activity represents the bulk of activity, so it is possible to characterize it in microsomal preparations. Using such preparations, we have examined the effects of anions on $H^+$ pumping and on the steady state pH component of $\Delta \psi$ ($\Delta \psi^++(\Delta \psi^- = -(2.303 \, RT/F) \Delta \phi + \Delta \psi$) generated by the $H^+$ pump after the addition of ATP. We present evidence that there is no specific $Cl^-$ channel; NO3- and Br- are more effective as counterions. The selective permeability of the membrane to various anions may be of importance in defining the physiological function of the tonoplast membrane.

MATERIALS AND METHODS

Soybean seeds (Glycine max L. cv Williams '79) were surface sterilized with 0.5% w/v NaClO and about 1% v/v ethanol, rinsed thoroughly, sown between two sheets of germination paper (Anchor Paper, St. Paul, MN) in trays covered with aluminum foil, and germinated at 25°C in the dark.

ATP (Boehringer Mannheim, W. Germany) was prepared as the bis-tris-propane salt using Dowex 8% cross-linked (Sigma) for ATPase assays. Na2ATP was prepared as a 0.5 m stock and pH adjusted to 6.5 with solid bis-tris-propane for proton pumping assays. All other chemicals were of reagent grade.

Microsomal Suspension Preparation. Three-d-old seedling root tips (about 5 mm long) were excised into aerated distilled deionized H2O. They were collected, rinsed, and then homogenized in a volume of chilled grinding medium 5 times their weight. The grinding medium consisted of 0.25 m sucrose, 10 mM EGTA and MgSO4, 25 mM Mes, 2.5 mM DTT, 10% v/v methanol and glycerol, pH adjusted to 7.0 with KOH; 1% w/v BSA was added prior to homogenization. The medium was prepared from stock solutions (except for DTT) or made fresh within 2 h of when it was used. The roots were homogenized with a polytron (Brinkmann Instruments, Westbury, NY) using a 5-s pulse at setting 8. The homogenate was filtered through six layers of cheesecloth and centrifuged at 13,000g for 6 min in a Beckman 50.2 rotor. The supernatant was recentrifuged as above. The resulting supernatant was centrifuged at 85,000g for 20 min. The pellet was resuspended at about 5 to 10 mg/ml protein concentration in either grinding medium or a suspension medium consisting of 0.25 m sucrose, 2.5 mM Mes and DTT, 10% v/v methanol and glycerol, pH adjusted to 7.0 with solid bis-tris-
Results and Discussion

The rate of quenching is linear for protein concentration as shown in Fig. 1. The total extent of quenching for Protein A was found to be 40% at a 10 mM concentration. Protein A was found to be the most sensitive to changes in pH and temperature.

Figure 2 shows the effect of sorbitol concentration on quenching and the proton leakage. The addition of 1 M sorbitol reduced the initial rate of quenching by 10%. The addition of 2 M sorbitol reduced the initial rate of quenching by 20%. The addition of 3 M sorbitol reduced the initial rate of quenching by 30%.

Discussion

The total extent of quenching for Protein A was found to be 40% at a 10 mM concentration. Protein A was found to be the most sensitive to changes in pH and temperature. The addition of 1 M sorbitol reduced the initial rate of quenching by 10%. The addition of 2 M sorbitol reduced the initial rate of quenching by 20%. The addition of 3 M sorbitol reduced the initial rate of quenching by 30%.

**Fig. 1.** Concentration dependence of initial rate of quenching and proton leakage. The initial rate of quenching and the proton leakage were measured at several concentrations of Protein A (0.1, 0.5, and 1.0 mM) and at several concentrations of Protein B (0.1, 0.5, and 1.0 mM). The initial rate of quenching was found to be linear with respect to the concentration of Protein A and Protein B.

**Fig. 2.** Effect of sorbitol concentration on quenching and the proton leakage. The addition of 1 M sorbitol reduced the initial rate of quenching by 10%. The addition of 2 M sorbitol reduced the initial rate of quenching by 20%. The addition of 3 M sorbitol reduced the initial rate of quenching by 30%.

**Table 1.** Initial rate of quenching and proton leakage at several concentrations of Protein A and Protein B.

<table>
<thead>
<tr>
<th>Protein A (mM)</th>
<th>Protein B (mM)</th>
<th>Initial Rate of Quenching (pM/min)</th>
<th>Proton Leakage (pM/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.1</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>30</td>
<td>15</td>
</tr>
</tbody>
</table>

**Table 2.** Initial rate of quenching and proton leakage at several concentrations of Protein A and Protein B.

<table>
<thead>
<tr>
<th>Protein A (mM)</th>
<th>Protein B (mM)</th>
<th>Initial Rate of Quenching (pM/min)</th>
<th>Proton Leakage (pM/min)</th>
</tr>
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<tbody>
<tr>
<td>0.1</td>
<td>0.1</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>30</td>
<td>15</td>
</tr>
</tbody>
</table>
Table 1. Effect of Sorbitol Concentration on the Proton Leak

Parameters from the experiment shown in Figure 2 are presented here. Initial rate of quenching was measured after the addition of ATP. The total extent of quenching was measured after the quenching had reached a steady state. After the addition of EDTA to inhibit the ATPase, the initial rate of recovery was determined using the final fluorescence after EDTA addition as the maximum fluorescence \( f_0 \). Half times were measured as the time required for half of total fluorescence recovery to occur.

<table>
<thead>
<tr>
<th>Sorbitol (mM)</th>
<th>Initial Rate of Quenching (% min(^{-1}))</th>
<th>Total Extent of Quenching (%)</th>
<th>Initial Rate of Recovery (% min(^{-1}))</th>
<th>( t_{1/2} ) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.3</td>
<td>26.2</td>
<td>5.1</td>
<td>1.5</td>
</tr>
<tr>
<td>125</td>
<td>11.4</td>
<td>35.1</td>
<td>5.5</td>
<td>2.2</td>
</tr>
<tr>
<td>250</td>
<td>13.2</td>
<td>36.7</td>
<td>6.7</td>
<td>2.1</td>
</tr>
<tr>
<td>500</td>
<td>10.7</td>
<td>47.9</td>
<td>9.1</td>
<td>3.2</td>
</tr>
<tr>
<td>1000</td>
<td>7.6</td>
<td>50.6</td>
<td>8.4</td>
<td>4.3</td>
</tr>
</tbody>
</table>

![Fig. 3. Nitrate inhibition of quenching and ATPase activity—concentration dependence. Quenching. Microsomes (100 \( \mu \)g protein) were incubated in fluorescence assay buffer (containing 50 mM KCl) with various concentrations of KNO\(_3\) for 6 min. Quenching of quinacrine fluorescence was initiated by adding 5 mM Na\(_2\)ATP. The initial rate of quenching with 50 mM KNO\(_3\) decreased from 3.7% min\(^{-1}\) to 2.6% min\(^{-1}\) when KCl was absent. ATPase. Microsomes (20 \( \mu \)g protein) were incubated at 30°C for 20 min in the presence of 50 mM KCl and various concentrations of KNO\(_3\). Gramicidin was added at a final concentration of 0.6 \( \mu \)M.](image)

![Fig. 4. Fluoride inhibition of quenching and ATPase activity—concentration dependence. Quenching. Microsomes (100 \( \mu \)g protein) were incubated in fluorescence assay buffer (containing 50 mM KCl) with various concentrations of NaF. Quenching of quinacrine fluorescence was initiated by adding 5 mM Na\(_2\)ATP. Two experiments (\( \bullet \), \( \square \)) are shown. ATPase. The experiment was performed as noted in Figure 3. The inhibition of color development using the Ames assay (1) by fluoride at concentrations greater than 20 mM has not been corrected for.](image)

![Fig. 5. Interaction between fluoride and nitrate inhibition of quenching. Microsomes (100 \( \mu \)g protein) were incubated in fluorescence assay buffer containing 50 mM KCl (control), 10 mM NaF and 50 mM KCl (NaF), 50 mM KNO\(_3\) replacing KCl (NO\(_3\)^{-}), 50 mM KNO\(_3\) and 10 mM NaF (NaF + NO\(_3\)^{-}). Quenching of quinacrine fluorescence was initiated by adding 5 mM Na\(_2\)ATP.](image)
ANION PERMEABILITY OF TONOPLAST-DERIVED VESICLES

Fig. 6. KCl- and KNO3-induced quenching. Potassium salts (50 mM KCl or KNO3) were added after quenching of quinacrine fluorescence in the absence of potassium salt had reached a steady state. Microsomes (200 μg protein) were suspended in fluorescence assay buffer without potassium salt. Quenching was initiated by the addition of 5 mM Na2ATP (first arrow). After 15 min, 75 μl of 1 M KCl or KNO3 was added (second arrow). A, Control; B, microsomes incubated in fluorescence assay buffer containing 10 μM DIDS.

Table II. Effect of Cl− and NO3− on the ΔpH of Microsomal Vesicles after Steady State Equilibration in the Absence of Potassium Salts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial Rate of Quenching (μM min−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.3</td>
</tr>
<tr>
<td>+ KCl</td>
<td>76</td>
</tr>
<tr>
<td>+ 500 mM Sorbitol</td>
<td>110</td>
</tr>
</tbody>
</table>

Fig. 7. Anion-induced quenching. The experiment was performed as noted in Figure 6. All salts were added as aliquots from 1 M stocks to a final concentration of 50 mM. Rates of quenching (% min−1) are shown.

The initial rate of quenching and a NO3−-sensitive ATPase in microsomal preparations (17). Soybean root microsomes exhibit a similar behavior (Fig. 3); the concentration dependence of inhibition of the initial rate of quenching and of the ATPase activity is quite similar. Even though the proton pumping activity is predominantly NO3−-sensitive, the NO3−-sensitive ATPase is a small component of total microsomal ATPase activity (18% of total activity), but is stimulated markedly by the addition of gramicidin (increasing to 35% of total activity), which presumably relieves inhibition due to 'stalling' at high ΔμH+.

Fluoride has relatively little effect on the initial rate of fluorescence quenching relative to its inhibition of microsomal ATPase (Fig. 4). The inhibition of fluorescence quenching by NaF occurs only when NO3− is present (Fig. 5). Vanadate also inhibits only when NO3− is present (13). These results are consistent with previous evidence that F− selectively inhibits vanadate-sensitive ATPase activity (12). This selectivity is dependent on the use of a relatively low concentration of F− (10 mM) which is more inhibitory than vanadate (100 μM) in the presence of NO3−.

Although NO3− inhibits the proton pumping ATPase, it can still act as a permeant anion. To examine this, it is necessary to set up a steady state ΔμH+ across the vesicle membranes which is dominated by ΔΨ. This can be done by adding ATP with no accompanying potassium salt. When fluorescence quenching reaches a steady state, the addition of a potassium salt causes a rapid increase in the extent of fluorescence quenching. This is caused by the electrophoresis of the anion into the vesicle interior, alleviating the ΔΨ and increasing the ΔpH component of ΔμH+ (Fig. 6). The addition of the potassium cation may stimulate the H+-ATPase to some extent; for example, NaCl causes slightly less quenching than KCl.

The rate of quenching under these conditions can be used to determine the relative permeabilities of the vesicle membranes to anions. Although the anion influxes were not measured, the subsequent measurable increases in H+ influx will depend on the relative anion influxes. Thus, relative anion permeabilities can be determined. NO3− is more permeable than Cl− under these conditions (Fig. 6). The permeability properties were also examined using step gradient-purified, NO3−-sensitive proton pumping activity (20%/34% w/w interface on sucrose density step gradients) and similar results were observed. After the initial anion-induced quenching, the inhibition by NO3− causes an apparent proton leak (Fig. 6). Based on these observations, it became clear that previous evidence for a Cl− channel could be reexamined in a more direct fashion. The addition of the anion channel blocker DIDS caused only a slight attenuation of the rate of quenching caused by either KCl or KNO3 (Fig. 6B; Table II). Sorbitol at 500 mM causes even greater attenuation. Since 500 mM sorbitol causes a decrease in the proton leak, one would also expect a decrease in the permeability of other ions. This
implies that the passive permeability properties of the vesicles are an important component of the ability of various anions to alleviate $\Delta \Psi$. When DIDS is added with 50 mm KCl prior to the addition of ATP, there is a slight inhibition of the initial rate of quenching (0–20%).

Similar experiments were performed with 3-d-old corn seedling roots. The results were nearly the same as for those using soybean. Rates of quenching induced by 50 mm KCl and KNO$_3$ were 66% min$^{-1}$ and 119% min$^{-1}$, respectively, in the absence of DIDS, and 57% min$^{-1}$ and 78% min$^{-1}$, respectively, in the presence of 10 mm DIDS. The initial rate of quench in the presence of 10 mm DIDS and 50 mm KCl was inhibited about 32%; this is comparable to the results of Bennett and Spanswick (2). DIDS is known to inhibit the Cl$^-$-stimulated ATPase even in the presence of gramicidin (2); this may be the reason for its inhibition of anion-induced quenching.

The ability of both KCl and KNO$_3$ to induce quenching and the slight attenuation of anion-induced quenching by DIDS indicate that there is no specific Cl$^-$ channel dominating the permeability properties of the vesicle membranes in either corn or soybean. Passive permeability is an important component of anion movement, but the presence of an anion channel is possible, based on anion specificity and concentration dependence of anion-induced quenching.

The permeabilities of other anions (as potassium salts, 50 meq A$^{-}$) were examined as shown in Figure 7. Br$^-$, NO$_3^-$, and Cl$^-$ are quite permeable, while SO$_4^{2-}$ has little effect. Phosphate (at pH 6.5) caused a slight increase in fluorescence, which then remained stable at meq phosphate levels of 50, 25, or 10 mm. Fluoride had a similar effect (data not shown).

The ability of Br$^-$, NO$_3^-$, and Cl$^-$ (but not SO$_4^{2-}$ or phosphate) to alleviate the $\Delta \Psi$ was confirmed using oxonol V as a probe of the $\Delta \Psi$ (Fig. 8). ATP-dependent quenching of oxonol V fluorescence was measured as in Figure 7. The quenching (indicative of an inside positive potential) was completely reversed by the protonophore FCCP (data not shown) and almost completely reversed by Br$^-$, NO$_3^-$, or Cl$^-$/SO$_4^{2-}$ and phosphate had no significant effect.

The concentration dependence of KCl- and KNO$_3$-induced quinacrine fluorescence quenching was also examined (Fig. 9). Quenching exhibits a saturable component. Similar experiments were performed in the presence of 10 mm DIDS or 500 mm sorbitol. The rate of anion-induced quenching was lower, but the quenching still exhibited a saturable component, similar to the results shown in Figure 9.

The presence of a saturable component of anion-induced quenching suggests that an anion channel may be present in these membranes. There is also a marked specificity: SO$_4^{2-}$ causes very little quenching and, although the halides Cl$^-$ and Br$^-$ are effective, the halide F$^-$ is not. This specificity is consistent with the presence of an anion channel.

The possibility that the surface potential associated with these vesicles might affect anion permeability was also examined. The addition of 50 or 5 meq Cl$^-$ as MgCl$_2$/MgSO$_4$ or MgCl$_2$ caused slower quenching than KCl, even though the divalent cation should ‘shield’ the surface potential and allow anions to approach and permeate the membrane more readily. Thus surface potential effects appear to be unimportant.

Three of the anions examined have an important physiological role. Chloride is a common counterion and may be actively sequestered in plant cell vacuoles (19). There is evidence that
nitrate can be stored in some cellular compartment (9), presumably the vacuole (15). Although metabolic pools of phosphate exist in mitochondria, the only evidence for its presence in vacuoles is based upon 31P NMR studies where a phosphate signal having a chemical shift consistent with an acidic environment has been reported for root tissue (21) and characterized in terms of aging and metabolic inhibitor effects (11).

Based upon relative permeabilities, it is apparent that both Cl- and NO3- may be 'driven' into the vacuole by the activity of the tonoplast proton pumping ATPase. The Cl- stimulation of the pump is probably physiologically significant. The ability of NO3- to permeate more rapidly than Cl- suggests that it as well may be electrophoresed in under in vivo conditions; the inhibition of the pump at concentrations greater than 1 mm may be a regulatory factor with physiological significance. One likely regulatory function involves pH control: nitrate reduction to NH4+ consumes 8 H+ (10 H+ if incorporation into amino acids is considered). If nitrate levels are higher than about 1 mm, inhibition of the tonoplast H+-ATPase will cause reversal of vacuolar acidification, relieving the cytoplasmic alkalization caused by nitrate reduction. Phosphate, on the other hand, does not electrophores into the vesicles, suggesting that sequestration in the vacuole may occur by some other mechanism, such as movement into the vacuole as a phosphorylated sugar with subsequent cleavage of the ester linkage. We tested the effect of glucose-6-phosphate, which is present in the cytoplasm at relatively high levels (21), on the steady state quench. It caused only slightly enhanced quenching in a manner similar to SO4, indicating that if it is taken up, the uptake is not coupled to either the Δψ or the pH gradient in these vesicles.

Acknowledgment—We would like to thank Dr. David S. Perlin (Department of Biochemistry, University of Rochester Medical Center, Rochester, NY) for discussion and helpful suggestions.

LITERATURE CITED


APPENDIX

The assumption that the equilibration of the pH gradient is described by an exponential function is based on the following:

We assume that Δψ is constant (KCl is present); thus, we can use the equation for the flux of a neutral solute:

\[ J_{\text{net}} = P_H (H^+ - H^+) \]  

(1a)

where \( H^+ \) and \( H^+ \) are the internal and external concentrations, respectively, and \( P_H \) is the permeability of the membranes to \( H^+ \). \( J_{\text{net}} \) is also described by:

\[ J_{\text{net}} = \frac{V}{A} \frac{dH^+}{dt} = P_H (H^+ - H^+) \]  

(2a)

Since the external volume is very large relative to the internal volume of the vesicles and is buffered, we can assume that \( H^+ \) will remain constant. If \( H^+ = [H^+]_0 \) at \( t = 0 \) and \( H^+ = [H^+]_0 \) at \( t = t \), we can integrate to give the solution:

\[ H^+ = [H^+]_0 + [\Delta H^+]_0 \exp \left[-\frac{A}{V} P_H t\right] \]  

(3a)

where \( [\Delta H^+]_0 = [H^+]_0 - H^+ \).

The halftime is given by:

\[ t_0 = 0.693 \frac{V}{A} P_H \]  

(4a)

If we examine the effect of volume change on the \( t_0 \), we find that it is proportional to the inverse of the cube root of the osmotic concentration, assuming the vesicles act as perfect osmometers. Since \( V \propto 1/\text{osmotic concentration} \), and the term \( V/A \) in Eq. 4a is equal to \( r/3 \) for spherical vesicles, then \( 4/3 \propto 1/\text{osmotic concentration} \) so that

\[ t_0 \propto 1/\text{osmotic concentration} \]  

(5a)

Equation 5a suggests that increasing osmotic concentration will cause a decrease in the \( t_0 \) if \( P_H \) is constant. In fact it increases (Table 1), implying that \( P_H \) itself is decreasing.

We are not measuring \( H^+ \) directly, but the %Q. For this reason, it is the plot of %Q(100 – %Q) versus time which should be an exponential function. We tested this for some of the data shown in Figure 2. The \( r^2 \) values for an exponential fit were greater than 0.9, and greater than \( r^2 \) values for plots of %Q versus time.