Mechanism of Stimulation and Inhibition of Tonoplast H⁺-ATPase of Beta vulgaris by Chloride and Nitrate

Christopher J. Griffith, Philip A. Rea, Eduardo Blumwald, and Ronald J. Poole

Department of Biology, McGill University, 1205 Avenue Docteur Penfield, Montreal, Quebec, Canada H3A 1B1

Received for publication October 8, 1985 and in revised form January 23, 1985

ABSTRACT

The H⁺-ATPase of tonoplast vesicles isolated from red beet (Beta vulgaris L.) storage tissue was studied with respect to the kinetic effects of Cl⁻ and NO₃⁻. N-Ethylmaleimide (NEM) was employed as a probe to investigate substrate binding and gross conformational changes of the enzyme. Chloride decreased the Kₐ of the enzyme for ATP but caused relatively little alteration of the Vₘₐₓ. Nitrate increased Kₐ only. Michaelis-Menten kinetics applied throughout with respect to ATP concentration. Nitrate yielded similar kinetics of inhibition in both the presence and absence of Cl⁻. Other monovalent anions that specifically increased the Kₐ of the ATPase for ATP were, in order of increasing Kₐ, SCN⁻, ClO₄⁻, and ClO₃⁻. Sulfate, although inhibitory, manifested noncompetitive kinetics with respect to ATP concentration. ADP, like NO₃⁻, was a competitive inhibitor of the ATPase but ADP and NO₃⁻ did not interact cooperatively nor did either interfere with the inhibitory action of the other. It is concluded that NO₃⁻ does not show competitive kinetics because of its stereochemical similarity to the terminal phosphoryl group of ATP. NEM was an irreversible inhibitor of the tonoplast ATPase. Both Mg-ADP and Mg-ATP protected the enzyme from inactivation by NEM but Mg-ADP was the more potent of the two. Chloride and NO₃⁻ exerted little or no effect on the protective actions of Mg-ADP and Mg-ATP suggesting that neither Cl⁻ nor NO₃⁻ are involved in substrate binding.

Electrogenic H⁺ translocation is thought to be the primary transport event across the plasma and vacuolar membranes of plant cells (18). A pH gradient (inside-acid versus cytoplasm) and sometimes an electrical potential difference (inside-positive versus cytoplasm) can be measured across the vacuolar membrane (tonoplast) of intact cells (10). Together with the demonstration of ATPase activity and ATP-dependent electrogenic H⁺-translocation into isolated vacuoles, these findings have established the existence of a H⁺-translocating ATPase in tonoplast (19).

With the development of techniques for the preparation of sealed membrane vesicles from various plant tissues, several laboratories have demonstrated an electrogenic H⁺-ATPase associated with low density microsomes (15). The low density microsomes are identified as tonoplast according to three primary criteria: (a) vesicles prepared from intact isolated vacuoles equilibrate at the same density (about 1.10 g/ml) upon density centrifugation and show similar enzyme (20) and H⁺-transloca-

---

1 Supported by the Natural Sciences and Engineering Research Council of Canada and the Department of Education of Quebec.

2 Address for correspondence: Department of Biology, University of York, Heslington, York Y01 5DD, England.
Tris-Mes (pH 8.0), 3 mM MgSO₄, 3 mM PEP, 0.184 mM NADH, 2 units pyruvate kinase, and 1 unit lactate dehydrogenase. The reaction was initiated by the addition of the desired concentration of Tris-ATP. Whenever KCl was omitted from the incubation medium, it was replaced by an equivalent concentration of K-Mes to ensure that the overall reaction was not rate-limited by pyruvate kinase which requires K⁺ as cofactor.

Five μM gramicidin D was included in all of the assay media to ensure that the H⁺-ATPase was not rate-limited by ΔpH and/or Δψ.

NEM Protection Experiments. The mechanisms of action of Cl⁻, NO₃⁻ and ADP on the tonoplast ATPase were examined by determining their capacity to alter the susceptibility of the enzyme to irreversible inhibition by the sulfhydryl reagent NEM. Since Mg-ATP and Mg-ADP protect the enzyme from inactivation by NEM (see "Results"), it was assumed that if Cl⁻ and NO₃⁻ effect substrate binding they should have an effect on the protective actions of Mg-ATP and Mg-ADP.

The DTT in the tonoplast suspension was removed by dilution of 250 μg membrane protein in 25 ml of 30 mM Tris-Mes (pH 8.0) containing 10% (w/v) glycerol ('glycerol medium'). The membranes were pelleted at 100,000g, resuspended in another 25 ml glycerol medium, pelleted again and resuspended in glycerol medium. Twenty-five μg membrane protein were added to 100 ml volumes of the NEM inhibition solutions (50 mM KCl, 1.5 mM MgSO₄, 30 mM Tris-Mes, pH 8.0, containing 1-25 μM NEM, unless otherwise indicated). After incubation for 5 min on ice, the reaction was terminated by the addition of 75 μl of 750 μM DTT. The samples were then centrifuged through 1 ml Sephadex columns equilibrated with 50 mM KCl, 1.5 mM MgSO₄, and 30 mM Tris-Mes buffer (pH 8.0) at 112g for 2 min. Two hundred μl volumes of the Sephadex-treated tonoplast suspensions were assayed for ATPase activity by the Ames method.

Protein. Protein was estimated as described previously (4).

Chemicals. General laboratory reagents were obtained from BDH Chemicals, Canada, Ltd., Boehringer-Mannheim, Canada, and Sigma Chemical Co. [γ-³²P]ATP (3-4 Ci/mmole) was a kind gift from Dr. Rhoda Blostein, Montreal General Hospital.

Fig. 1. Influence of Cl⁻ on ATP concentration-dependence of tonoplast ATPase. Main figure: plot of 1/v against 1/ATP at Cl⁻ concentrations of 0, 5, 10, 25, and 50 mM, respectively. Inset: effect of Cl⁻ on apparent Km and Vmax. ATPase activity was measured by coupling ATP hydrolysis to NADH oxidation as described in "Materials and Methods."

2 Abbreviations: PEP, phosphoenolpyruvate; NEM, N-ethylmaleimide; ΔpH, pH difference (outside-inside, where pH = -log [H⁺]); pmf, proton motive force; Δψ, electrical potential difference (inside-outside).

Fig. 2. Influence of NO₃⁻ (A) and other anions (B) on ATP concentration-dependence of tonoplast ATPase in presence of 50 mM KCl. Nitrates were present at the concentrations indicated (A). Thiocyanate (Δ), CIO₂⁻ (△), Cl⁻O₃⁻ (○) and CH₃COO⁻ (■) were present at 50 mM, while SO₄²⁻ (■) was present at 25 mM; control activity (E) (B). All of the anions were added as their K⁺-salts. Conditions otherwise identical to Figure 1.

Fig. 3. Secondary plot of Km_ATP against anion concentration for anions examined in Figure 2. Nitrates, SCN⁻, ClO₃⁻ and ClO₂⁻ yield Kₘ values of 21.9, 38.3, 54.7, and 120 mM, respectively.
RESULTS

Kinetics of Activation by Chloride and Inhibition by NO₃⁻.

The activity of the tonoplast ATPase approximated Michaelis-Menten kinetics with respect to ATP concentration in both the presence and absence of Cl⁻ (Fig. 1). Providing that uncoupler (5 μM gramicidin D) was included in the assay media, the predominant effect of Cl⁻ on catalysis was to decrease the $K_m$ of the enzyme for ATP (Fig. 1 and inset).

Nitrate increased the $K_m$ of the enzyme for ATP (Fig. 2A). Michaelis-Menten kinetics with respect to ATP concentration were approximated at all of the NO₃⁻ concentrations employed and a secondary plot of the results yielded a straight-line relationship with a $K$ value of 21.9 mM (Fig. 3). Chlorate, a putative analog of NO₃⁻ (6) was markedly less effective as an inhibitor but yielded competitive kinetics ($K_I$ about 120 mM; Figs. 2B and 3).

The data shown correspond to those in Table I. B, Arithmetic plot of ATPase activity against ATP concentration. Activities in the absence of ADP and NO₃⁻ (○); activities in the presence of 10 mM NO₃⁻ (▲), 100 μM ADP (■), and 100 μM ADP + 10 mM NO₃⁻ (△), respectively. The theoretical relationship between activity and ATP concentration (△) was calculated by assuming that the inhibitions exerted by 100 μM ADP and 10 mM NO₃⁻, individually, interact in a simple additive manner (see Table I for method of calculation). Fifty mM KCl were present throughout and ATPase activity was measured by monitoring the release of $^{32}$Pi from $^{[\gamma-32P]}$ATP.
Table 1. Influence of Different Combinations of ADP and NO3− on Activity of Tonoplast ATPase

<table>
<thead>
<tr>
<th>Effector</th>
<th>Concentration (mm)</th>
<th>ATPase Activity (μmol/mg protein·h)</th>
<th>Percent of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td></td>
<td>10.53</td>
<td>100.0</td>
</tr>
<tr>
<td>Nitrate</td>
<td>0.05</td>
<td>8.10</td>
<td>76.9</td>
</tr>
<tr>
<td>ADP</td>
<td>0.05</td>
<td>8.10</td>
<td>76.9</td>
</tr>
<tr>
<td>ADP + nitrate (experimental)</td>
<td>0.05:25</td>
<td>5.33</td>
<td>50.6</td>
</tr>
<tr>
<td>ADP + nitrate (theoretical)</td>
<td>0.05:25</td>
<td>5.08</td>
<td>48.2</td>
</tr>
</tbody>
</table>

ATP, Mg2+ and KCl were present at concentrations of 0.15, 1.5, and 50 mm, respectively. The theoretical activities for different combinations of ADP and NO3− were calculated by assuming that the inhibitions exerted by ADP and NO3− in combination were equivalent to the sum of their inhibitions in isolation: theoretical activity, μmol/mg·h = (% control activity ADP) × (% control activity NO3−) × control activity, μmol/mg·h.

Inhibition of Basal ATPase Activity. The kinetics of inhibition observed above (Figs. 2 and 3) were obtained in the presence of 50 mm KCl. Similar kinetics of inhibition by NO3− were also obtained in the absence of KCl (Fig. 4). The Ki value obtained in the absence of Cl−-stimulation (38.5 mm; Fig. 4) was approximately 1.8-fold greater than that obtained in the presence of Cl− (21.9 mm above) suggesting that the Cl−-stimulated component of ATPase activity is more sensitive to inhibition by NO3− than the basal component.

Lack of Interaction between NO3− and ADP. ADP, like NO3−, is a competitive inhibitor of the ATPase and yields a Ki value of 0.12 mm (Fig. 5). However, ADP and NO3− neither interact cooperatively nor negatively during inhibition. The competitive effects of ADP and NO3− were additive (Fig. 6 and Table I). The inhibitions found when ADP and NO3− were present together were similar to the theoretical sum of the inhibitions obtained with each inhibitor alone, where theoretical activity, μmol/mg·h = (% control activity with ADP) × (% control activity with NO3−) × control activity, μmol/mg·h.

Protection from Irreversible Inhibition by NEM. NEM, a sulfhydryl reagent, inhibited the tonoplast ATPase with a k50 of 2.5 μM but the inclusion of Mg-ADP or Mg-ATP in the NEM-inhibition solutions significantly decreased the degree of inhibition exerted (Fig. 7A). Mg-ADP was more effective than Mg-ATP as a protectant and ADP and ATP, alone, were less effective than their corresponding Mg-salts (Fig. 7A). The protective actions of Mg-ADP and Mg-ATP indicate the presence of essential sulfhydryl groups at the active site of the ATPase whereas the greater efficacies of Mg-ADP and Mg-ATP over ADP and ATP alone suggest that Mg2+ is required for efficient nucleotide binding.

Neither Cl− nor NO3− had a marked effect on the protective actions of Mg-ADP and Mg-ATP (Figs. 7B and 8A). The most marked anion effect observed was a decrease in the protective action of Mg-ADP at low NEM concentrations when Cl− was included in the incubation media (Fig. 7B). It was also found that Cl− and NO3−, alone and together, enhanced the inhibitory
effect of NEM over that seen in their absence whereas SO₄²⁻ had a protective action (Fig. 8B). Anions are apparently capable of increasing or decreasing the accessibility of essential sulphydryl groups on the ATPase but do not appear to influence substrate binding.

**DISCUSSION**

In the experiments described above the predominant effect of Cl⁻ was to decrease the $K_m$ of the tonoplast ATPase for ATP (Fig. 1). While several authors (3, 5, 7, 21) have found that Cl⁻ primarily affects the $V_{max}$ component of ATPase activity, to our knowledge, none have examined the effect of Cl⁻ on the ATP concentration-dependence of catalysis in uncoupled tonoplast vesicles where complications due to $\Delta$pH and $\Delta$ψ are excluded. Permeant anions, such as Cl⁻, alleviate the development of an inside-positive $\Delta$ψ upon H⁺ translocation and thereby increase the $\Delta$PH component of the pmf (3, 9). And, as shown by the stimulatory effects of ionophores, such asgramicidin D, nigericin or $m$-chlorocarboxymethyl phenylhydrazine in combination with K⁺-valinomycin, on ATPase activity (14), ATP-dependent H⁺ translocation is subject to stalling by the pmf developed during the course of translocation. Thus, providing that the magnitude of the pmf is the determining factor (i.e. the vesicles are tightly coupled), the diminution of $\Delta$ψ by the influx of counter-anions will cause a corresponding increase in the rate of H⁺ translocation. Consequently, it would be expected that in the coupled condition the principal effect of Cl⁻ is to increase pump turnover, and thence $V_{max}$, even if Cl⁻ decreases $K_m$ in the uncoupled condition, as found here.

The mechanism of action of Cl⁻ remains elusive. The NEM protection experiments indicate that Cl⁻ is not required for Mg-ADP or Mg-ATP binding in so far as Cl⁻ does not facilitate the protective action of either nucleotide (Fig. 7). It therefore appears that the influence of Cl⁻ on the $K_m$ of the ATPase for ATP is exerted at some point in the catalytic cycle after substrate binding. However, interpretation of the NEM protection experiments is complicated by the fact that Cl⁻ or NO₃⁻ alone, increase the sensitivity of the enzyme to NEM over that seen in their absence, whereas SO₄²⁻ has a protective action (Fig. 8A).

It has been known for some time that the H⁺-ATPase of tonoplast is inhibited by NO₃⁻ (7, 20) but its mode of action is unknown. Although it has previously been shown that ADP is a competitive inhibitor of the tonoplast ATPase (Ref. 2 and Fig. 5), our demonstration of competitive kinetics for NO₃⁻ has not been reported before (Figs. 2–4). However, NO₃⁻ and ADP do not appear to have similar mechanisms of action. Mg-ADP protected the ATPase from inhibition by NEM but NO₃⁻ exerted no protective action (Fig. 8). The protective actions of Mg-ADP and Mg-ATP are consistent with the presence of essential sulphydryl groups at the active site of the ATPase which are rendered inaccessible to NEM by substrate or product. By the same token, the lack of protection by NO₃⁻ is consistent with the interaction of this anion with a site distinct from the substrate binding site of the enzyme.

At the outset of this investigation we postulated that NO₃⁻ exerts its effect in a competitive manner relative to ATP because of its trigonal planar geometry and therefore its structural similarity to the terminal phosphoryl group of ATP, by analogy with the mechanism proposed for the inhibition of creatine kinase by NO₃⁻ (13). According to the scheme proposed by Milner-White and Watts (13) for creatine kinase, Mg-ADP and NO₃⁻ can both occupy the active site to form a dead-end complex that stereochemically simulates the transition-state complex of Mg-ATP. In agreement with their scheme, NO₃⁻ in combination with Mg-ADP potentiated the protection of creatine kinase from inhibition by the sulphydryl reagent iodoacetamide. We, however, could not find any evidence for such a mechanism for the tonoplast ATPase. Nitrate and ADP did not cooperatively inhibit ATP hydrolysis (Table 1 and Fig. 6) and NO₃⁻ did not potentiate the protective action of Mg-ADP against inhibition by NEM (Fig. 8).

All of the anions capable of increasing the $K_m$ of the ATPase for ATP (SCN⁻, ClO₄⁻ and NO₃⁻) are chaotropic whereas those which do not yield competitive kinetics (CH₃COO⁻ and SO₄²⁻) are antichaotropic (Figs. 2 and 3). Chaotropes decrease water structure and, in agreement with the concept that the apolar groups of macromolecules form hydrophobic bonds because of their thermodynamically unfavorable interaction with water, the relative water structure-breaking properties of chaotrophic anions are correlated with their ability to destabilize membranes and enzyme complexes (8). Recent investigations demonstrate that the tonoplast ATPase is a multimeric complex comprising at least three different polypeptides (11, 12) and preliminary experiments show that this phosphohydrodase is markedly more sensitive to irreversible inhibition by 0.3 to 0.9 M concentrations of chaotropic anions than the tonoplast PPase (PA Rea, C. Bunyon, 124 125).
CJ Griffith, RJ Poole, unpublished data). Since the tonoplast PPase is not subject to reversible inhibition by millimolar concentrations of NO$_3^-$ (16, 17), it is possible that reversible inhibition of the ATPase by comparatively low concentrations of chaotropic anions and irreversible inhibition by high concentrations are related.

The reversible inhibitions exerted by the anions tested fall in the sequence NO$_3^-$ > SCN$^-$ > ClO$_4^-$ > CH$_3$COO$^-$ (Figs. 2 and 3), whereas their chaotropic potencies fall in the sequence SCN$^-$ > ClO$_4^-$ > NO$_3^-$ > CH$_3$COO$^-$ (8). Nitrate is ostensibly an anomalously potent inhibitor if chaotropism is the principal cause of the inhibitions seen. There are, however, numerous examples of weak chaotropes, such as NO$_3^-$, being more effective destabilizing agents than potent chaotropes (8). The chaotropic potency of anions is inversely related to their charge densities, whereas their capacity to destabilize electrostatic interactions is directly related to their charge density. Thus, if both hydrophobic and electrostatic interactions participate in the maintenance of enzyme functional integrity, the destabilizing effect of a weak chaotrope on both types of interaction might be greater, in balance, than that of a strong chaotrope. A chaotropic mechanism of this type could help explain the differential sensitivities of the ATPase and PPase to NO$_3^-$; irreversible inhibition of the ATPase but not the PPase by high concentrations of chaotropic anions; the capacities of SCN$^-$ and ClO$_4^-$, but not the NO$_3^-$-analog ClO$_4^-$, to simulate the effects of NO$_3^-$; the inhibitory effects of chaotropic anions but not neutral chaotropes (e.g. urea; data not shown); the sensitivities of both the Cl$^-$-stimulated and basal ATPase activities to NO$_3^-$; and the two-fold greater susceptibility of the solubilized, partially purified ATPase to NO$_3^-$ (12). We are currently investigating this hypothesis systematically.

LITERATURE CITED

6. DEANE-DRUMMOND CE, ADM GLASS 1982 Nitrate uptake into barley (Hordeum vulgare) plants. A new approach using $\text{ClO}_4^-$ as an analog for NO$_3^-$. Plant Physiol 70: 50–54
16. REA PA, RJ POOLE 1985 Proton translocating inorganic pyrophosphatase in red beet (Beta vulgaris L.) tonoplast vesicles. Plant Physiol 77: 46–52