Density gradient localization of vanadate- and NO$_3^-$-sensitive ATPase from sterile cultures of *Spirodea polyrrhiza* (L.) Schleiden

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Abstract

The present work deals with the separation and some characteristics of ATPase activities bound with plant membranes prepared from sterile cultures of *Spirodea polyrrhiza*. The membrane-bound ATPases were separated on sucrose gradients and distinguished by membrane density and sensitivity to several inhibitors. The results showed that NO$_3^-$-sensitive ATPase activity associated with the tonoplast was localized at a sucrose density between 1.095-1.117 g cm$^{-3}$. The vanadate-sensitive ATPase activity bound with the plasma membrane showed a density between 1.127-1.151 g cm$^{-3}$. Both ATPases were insensitive to azide and oligomycin and were separable from markers for mitochondria.

Key words: *Spirodea polyrrhiza* (L.) Schleiden, membrane-bound ATPase

INTRODUCTION

The existence of an ATPase which may mediate active proton transport and probably active ion transport across the plant plasma membrane (plasmalemma) and tonoplast has been detected by several authors (for review see Hodges 1976, Serrano 1983). The ATPases of both plasma membrane and tonoplast are K$^+$ sensitive and require Mg$^{2+}$ for activity (Leonard and Hotchkiss 1976, Churchill et al. 1983, O’Neill et al. 1983, Aoki and Nishida 1984). The most striking feature is their high specificity for ATP as substrate (Leonard and Hodges 1973, Benson and Tipton 1978, Leonard and Hotchkiss 1978, Bennett et al. 1984, Scherer 1984)

This paper demonstrates partial separation of two types of ATPases isolated from sterile cultures of S. polyrrhiza using continuous and discontinuous sucrose density gradients. The two types of ATPase can be distinguished by their relative densities and sensitivity to inhibitors.

MATERIAL AND METHODS

Plant material. Freshly harvested 14 day-old (exponential phase) Spirodea polyrrhiza (L.) Schleiden, were for all membrane preparations. Sterile cultures of S. polyrrhiza were grown in nitrate medium (Buczek 1984) under conditions of 16 h light (10.3 w m⁻², obtained from white fluorescent tubes) at 25 °C and 8 h darkness at 21 °C.

Membrane isolation. Approximately 40-50 g fresh weight of 14 day-old S. polyrrhiza culture were rinsed in cold water and homogenized using an ice-cold mortar and pestle. The homogenization medium consisted of 250 mM sucrose, 2.0 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% bovine serum albumin (BSA) and 25 mM Tris/Mes buffer (pH 7.3) with 4 cm³ homogenization medium per g fresh weight of tissue. The homogenate was strained through two layers of Miracloth and was centrifuged at 13000 × g for 10 min. The resulting supernatant was centrifuged at 80000 × g for 30 min. The 80000 × g pellet (crude membrane fraction) was resuspended in 250 mM sucrose, 1 mM DTT and 2.5 mM Tris/Mes buffer (pH 7.5) and used immediately either layered onto a 15%-45% (w/w) continuous sucrose gradient or 20%/30%/38% (w/w) discontinuous sucrose gradient and centrifuged in a SW 3 × 35 rotor and in a VAC 602 centrifuge at 80000 × g for 3 h. All gradient solutions contained 1 mM EDTA, 1 mM DTT and 2.5 mM Tris/Mes buffer (pH 6.5). Continuous or discontinuous gradient fractions were collected in 2 cm³ aliquots and assayed directly. For routine membrane preparations, membrane fractions were collected from 20%/30% (w/w) and 30%/38% (w/w) interfaces and repelletted at 80000 × g for 30 min. Pellets were resuspended in 250 mM sucrōse, 1 mM DTT and 2.5 mM Tris/Mes buffer (pH 6.5). All of the steps described above were carried out at a temperature of 0-4 °C.

Biochemical assays. ATPase activity was assayed for 30 min at 37 °C
in 2 cm³ reaction mixture containing 33 mM Tris/Mes buffer (pH 6.5), 3 mM ATP-Tris, 2.5 mM MgSO₄, 50 mM KCl and 25 mM K₂SO₄. For assaying ATPase activity in the presence of NO₃, 50 mM KNO₃ was added instead of 25 mM K₂SO₄. Modifications of the assay conditions are noted in the appropriate figure and table legend. Cytochrome c (Cyt. c) oxidase activity was assayed according Hodges and Leonard (1974) by measuring the oxidation of reduced Cyt. c at 550 nm. Protein was determined by the method of Markwell et al. (1978) using bovine serum albumin as the standard. Each value in the figures represents a mean of 3 replications and each experiment was repeated at least three times.

Source of chemicals. ATP was obtained from POCh-Poland as the disodium salt and was converted to the Tris salt by passage through Dowex 50 W. Sodium orthovanadate, DCCD (N,N'-dicyclohexylcarbodiimide) and Mes [2(N-morpholino)ethanesulfonic acid] from SIGMA, EDAC (ethyl-3-(3-dimethylaminopropyl carbodiimide)) and Tris [tris-(hydroxymethyl)aminomethane] from SERVA. All other chemicals were reagent grade.

RESULTS

Figure 1 presents the inhibition of ATPase activity of the crude membrane preparation (80000 g pellet) as the function of vanadate concentration, in the presence or absence of 50 mM KNO₃. ATPase activity in the presence of KNO₃ was lower than in its absence, which may indicate that there

![Fig. 1. The effect of NO₃⁻ on inhibition of ATPase activity by vanadate. Activity was assayed in the presence of 3 mM ATP, 2.5 mM MgSO₄, 50 mM KCl, 25 mM K₂SO₄, 33 mM Tris/Mes (pH 6.5) and 0.2 cm³ of crude membrane fraction (about 245 μg protein) and in the presence (●) or absence (○) of 50 mM KNO₃, with NO₃-sensitive ATPase (■) as the difference between the two. When KNO₃ was used, K₂SO₄ was omitted.](image-url)
are two ATPases in the crude membrane fraction. The increasing vanadate concentration in the reaction medium progressively inhibited the ATPase activity, both in the presence and absence of KNO₃. However, it seems that the degree of inhibition by NO₃⁻ (ΔNO₃⁻) did not change as the result of increasing vanadate concentration. This may indicate that vanad and NO₃⁻ inhibit different enzymes.

Total ATPase activity distribution on a continuous sucrose density gradient

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Fig. 2. Distribution of ATPase activity on a continuous sucrose density gradient. The total ATPase activity (A) was determined in the presence of 3 mM ATP, 2.5 mM MgSO₄, 25 mM K₂SO₄ and 50 mM KCl. The total activity of NO₃⁻-sensitive ATPase (ΔNO₃⁻)(B) was calculated as the difference between the total activity and that determined in the presence of 50 mM KNO₃ substituted for 25 mM K₂SO₄ in the reaction medium. The azide-sensitive ATPase activity (ΔN₃⁻)(B) was calculated as the difference between the total activity and that determined in the same medium with the addition of 2.5 mM NaN₃. The activity of NO₃⁻-sensitive ATPase assayed in the presence of 2.5 mM NaN₃ (Δ[NO₃⁻+N₃⁻]) (C) was calculated as the difference between the activity measured in the presence of KNO₃ with or without the addition of N₃. The activity of Cyto oxidase and sucrose percentage are also presented (A).
is shown in Fig. 2A, with two peaks occurring at 28% (w/w) sucrose and between 18 and 20% (w/w) sucrose. A peak of Cyt.c oxidase activity (Fig. 2A), a mitochondrial marker, occurred at 41% (w/w) sucrose and was almost associated with a major peak of $\Delta N_3$-sensitive (△$N_3$) ATPase

![Graph of ATPase activity on discontinuous sucrose density gradient.](image)

**Fig. 3.** Distribution of ATPase activity on discontinuous sucrose density gradient. Total (A), $\Delta$NO$_3$ and $\Delta N_3$ (B) and $\Delta[NO_3+N_3]$ (C) ATPase activities were assayed as previously (see Fig. 2) described. Vanadate-sensitive (△vanadate) ATPase (C) activity was calculated as the difference in ATPase activity assayed with 3 mM ATP, 2.5 mM MgSO$_4$, 50 mM KCl and in the absence or presence of 20 μM Na$_3$VO$_4$. Cyt.c oxidase is also presented (A).

(Fig. 2B). NO$_3$-sensitive ATPase (△NO$_3$) on linear sucrose density gradients showed two distinct peaks and one very low peak. The two distinct peaks were identified at 26-27% (w/w) sucrose and 22% (w/w) sucrose, while the lower third peak (at 39% (w/w)) was coincident with $N_3$-sensitive ATPase.
On the other hand, when NO$_3$-sensitive ATPase activity was assayed in the presence of N$_3^-$ (Fig. 2C) the distribution showed one peak between 20 and 24\% (w/w) sucrose with optimum activity at 22\% (w/w) sucrose. This was highly correlated with the distribution of ΔNO$_3$ ATPase (Fig. 2B).

The above results show that in the membrane preparations from *S. polyrrhiza* at least three ATPases exist. Partial separation of these ATPases was obtained with a discontinuous sucrose density gradient. From the gradients shown in Fig. 2 and similar ones presented by other authors (Bennett and Spanswick 1983, O’Neill et al. 1983) a three-step discontinuous sucrose density gradient of 20, 30 and 41\% (w/w) sucrose was chosen.

As indicated in Fig. 3, mitochondria clearly separated in the discontinuous sucrose gradient from the peak characteristic for membrane ATPases which

![Graph showing effect of pH on ATPase activity](image)

**Fig. 4.** Effect of pH on ATPase activity associated with low density (A) or high density (B) *S. polyrrhiza* membrane fraction. ATPase activity was assayed in the presence 3 mM ATP, 2.5 mM MgSO$_4$, 50 mM KCl and 33 mM Tris/Mes buffer. The assay pH was adjusted by varying the ratio of Tris/Mes.

is shown by the activity of Cyt.c oxidase (Fig. 3A) almost coincident with N$_3^-$-sensitive ATPase (Fig. 3B). However, the other peak of N$_3^-$-sensitive ATPase was observed at the region of 1.10-1.12 sucrose densities which was not correlated with Cyt.c oxidase activity. This activity may result from mitochondrial F$_1$-ATPase dislocated during isolation. NO$_3$-sensitive
Density gradient localization

ATPase showed two peak densities, one at 1.09 g cm\(^{-3}\) and the other at 1.11 g cm\(^{-3}\), however, only a lower density peak of NO\(_3\)-sensitive ATPase (Fig. 3B) coincided with the density peak of NO\(_3\)-sensitive ATPase measured in the presence of N\(^{-}\) (Fig. 3C. \(\triangle[NO_3^-+N^-]ATPase\)). Figure 3C shows the distribution of vanadate-sensitive ATPase activity which was associated with the lower density peak of total ATPase activity and was insensitive to NO\(_3\) and azide.

Based on the distribution of ATPase activity on discontinuous sucrose density gradient membrane enriched either NO\(_3\)-sensitive 23-28\(^\circ\)\_\(\text{w/w}\) sucrose (1.095-1.117 g cm\(^{-3}\)) or vanadate-sensitive 30-35\(^\circ\)\_\(\text{w/w}\) sucrose (1.127-1.151 g cm\(^{-3}\)) ATPase activity its may be separated. Both ATPases exhibit a pH optimum of 6.5 at 37 C (Fig. 4). The profile of vanadate-sensitive ATPase (30-35\(^\circ\)\_\(\text{w/w}\) sucrose) showed a sharper pH optimum than that of the NO\(_3\)-sensitive enzyme (23-25\(^\circ\)\_\(\text{w/w}\) sucrose).

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ATPase activity, (\mu\text{mol} \text{ P}_i \text{ mg}^{-1} \text{ protein} \cdot \text{ h}^{-1})</th>
<th>NO(_3)-sensitive ATPase activity of 23/28(^\circ)_(\text{w/w}) fraction</th>
<th>vanadate-sensitive ATPase activity of 30/35(^\circ)_(\text{w/w}) fraction</th>
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<tr>
<td>Control</td>
<td>4.7</td>
<td>100</td>
<td>7.5</td>
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<tr>
<td>DCCD, 100 (\mu\text{M})</td>
<td>1.2</td>
<td>25</td>
<td>2.1</td>
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<tr>
<td>EDAC, 1 (\text{mM})</td>
<td>4.3</td>
<td>92</td>
<td>5.8</td>
</tr>
<tr>
<td>Oligomycin, 5 (\mu\text{g cm}^{-3})</td>
<td>4.6</td>
<td>98</td>
<td>7.7</td>
</tr>
<tr>
<td>Oligomycin, 10 (\mu\text{g cm}^{-3})</td>
<td>4.4</td>
<td>93</td>
<td>7.3</td>
</tr>
<tr>
<td>Na(_3)VO(_4), 1 (\text{mM})</td>
<td>4.5</td>
<td>96</td>
<td>7.2</td>
</tr>
<tr>
<td>Na(_3)VO(_4), 20 (\mu\text{M})</td>
<td>4.3</td>
<td>92</td>
<td>1.9</td>
</tr>
<tr>
<td>KNO(_3), 50 (\text{mM})</td>
<td>1.4</td>
<td>30</td>
<td>6.8</td>
</tr>
</tbody>
</table>

ATPase activity was assayed in the presence of 3 \(\text{mM}\) ATP, 2.5 \(\text{mM}\) MgSO\(_4\), 50 \(\text{mM}\) KCl, 25 \(\text{mM}\) K\(_2\)SO\(_4\), 33 \(\text{mM}\) Tris-Mes buffer (pH 6.5) and 23/28\(^\circ\)\_\(\text{w/w}\), membrane fraction (about 36 \(\mu\text{g protein}\)) or 30/35\(^\circ\)\_\(\text{w/w}\), membrane fraction (about 38 \(\mu\text{g protein}\)). When KNO\(_3\) was used K\(_2\)SO\(_4\) was omitted.

Table 1 shows the effect of several inhibitors of ATPase activity associated with membranes collected from either 23-28\(^\circ\)\_\(\text{w/w}\) or 30-35\(^\circ\)\_\(\text{w/w}\) sucrose fractions. With respect to inhibitor sensitivities, both ATPases were highly sensitive to 100 \(\mu\text{M}\) DCCD and showed a marked differential sensitivity to vanadate and NO\(_3\). The low density ATPase was inhibited by 70\(^\circ\)\_\(\text{w/w}\) by 50 \(\text{mM}\) KNO\(_3\) and was weakly sensitive to 20 \(\mu\text{M}\) Na\(_3\)VO\(_4\). In contrast, the high density ATPase was inhibited by 75\(^\circ\)\_\(\text{w/w}\) by 20 \(\mu\text{M}\) Na\(_3\)VO\(_4\) and was almost insensitive to KNO\(_3\). Both ATPases were insensitive


to 1 mM azide and 5-10 μg·cm⁻³ oligomycin. The low density ATPase was insensitive to 1 mM EDAC, while this inhibitor weakly reduced the activity of vanadate-sensitive ATPase.

DISCUSSION

The present investigation shows that using the technique of centrifugation in sucrose density gradients it was possible to separate at least two distinct types of ATPases associated with microsomal (nonmitochondrial) fractions from *S. polyrrhiza*. The two types of ATPases can be separated with 20/30% (w/w) and 30/38% (w/w) discontinuous sucrose gradients. One type of ATPase, highly NO₃⁻-sensitive, was enriched in low density membranes and was found between 1.095-1.117 g·cm⁻³ (23-28% (w/w) sucrose) on a discontinuous sucrose density gradient. The other type of ATPase was found at a density between 1.127-1.151 g·cm⁻³ (30-35% (w/w) sucrose) on a discontinuous sucrose gradient and was highly vanadate-sensitive. Both types of ATPase were separable from markers for mitochondrial membranes.

The effect of specific inhibitors on ATPase activity associated with low or high density membranes from *S. polyrrhiza*, enabled some characteristics of the two different ATPases to be determined. Both ATPases were strongly inhibited by DCCD regarded as a nonspecific inhibitor of plant membrane-bound ATPase (Schoner and Schmidt 1969, Solioz 1984). Both ATPases were almost completely insensitive to oligomycin and azide, inhibitors of mitochondrial ATPase activity (Pedersen 1975, Stout and Cleland 1982, O’Neill et al. 1983), which may indicate that these membrane fractions were free from mitochondrial contaminations. The water-soluble carbodiimide EDAC had no effect on the NO₃⁻-sensitive ATPase, while vanadate-sensitive ATPase was inhibited by 23 per cent. This may suggest selective inhibition of the vanadate-sensitive ATPase from *S. polyrrhiza* which is in agreement with Walker and Leigh’s (1981) experiments on beet roots. However Bennett and Spanswick (1983), O’Neill et al. (1983) and Bennett et al. (1984) reported the opposite results with EDAC. This inhibitor had no effect on vanadate-sensitive ATPase, but they observed some inhibition of NO₃⁻-sensitive ATPase from corn roots.

On the other hand, the two ATPases from *S. polyrrhiza* showed a marked differential sensitivity to NO₃⁻ and vanadate. The fact that the activity of ATPase associated with low density membranes was highly NO₃⁻-sensitive and was insensitive to vanadate, azide and oligomycin, indicates that this type of ATPase is associated with the tonoplast. A similar characteristic of tonoplast ATPase was found by other authors for corn and beet roots (Admon et al. 1981, Perlin and Spanswick 1981, O’Neill et al. 1983, Bennett et al. 1984) but was not confirmed by Aoki and
Nishida (1984) for tonoplast ATPase from *Kolanchoë daigremontiana*. Inasmuch as the high density ATPase was strongly inhibited by vanadate, the lack of susceptibility of this ATPase to NO$_3^-$ shows that the vanadate-sensitive ATPase is associated with the plasma membrane (Perlin and Spanswick 1981, O’Neill et al. 1983, Bennett et al. 1984).

In addition to their differential sensitivities to inhibiton by NO$_3^-$ and vanadate, the high density or vanadate-sensitive ATPase showed a sharp pH optimum. This pH optimum 6.5 is characteristic of the plasma membrane ATPase (Briskin and Poole 1983, Bennett et al. 1984) suggesting that the vanadate-sensitive ATPase is in plasma membrane origin. The low density or NO$_3^-$-sensitive ATPase showed the same pH optimum 6.5, but the shape of pH profile of this ATPase was different. The NO$_3^-$-sensitive ATPase has a broad pH optimum which extends from pH 6.5 into the alkaline range. According to Bennett et al. (1984) this fact is characteristic only for NO$_3^-$-sensitive ATPase.

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REFERENCES


Lokalizacja, w gradiencie gęstości, ATPazy wrażliwej na wanad i NO₃ wyodrębnionej z sterylnych kultur Spirodea polyrrhiza

Streszczenie

W pracy przedstawiono próby rozdzielenia i charakterystyki ATPazy związanych z błonami, wypreparowanych z sterylnych kultur S. polyrrhiza. ATPazy rozdzielono w gradiencie gęstości sacharozy i rozróżniono na podstawie różnic gęstości błon oraz ich wrażliwości na niektóre inhibitory. Wyniki wskazują, że aktywność ATPazy wrażliwej na NO₃ związanej z tonoplastem występowała w gęstości sacharozy miedzy 1.095-1.117 g cm⁻³. Aktywność ATPazy wrażliwej na wanad, związanej z plazmalem, występowała w gęstości sacharozy miedzy 1.127-1.151 g cm⁻³. Obydwa ATPazy były niewrażliwe na azidek i oligomycynę i były oddzielone od obszaru występowania markera mitochondriów.