Density gradient localization of vanadate- and NO_3^- -sensitive ATPase from sterile cultures of *Spirodela 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 *Spirodela 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 $\text{g}\cdot\text{cm}^{-3}$. The vanadate-sensitive ATPase activity bound with the plasma membrane showed a density between 1.127-1.151 $\text{g}\cdot\text{cm}^{-3}$. Both ATPases were insensitive to azide and oligomycin and were separable from markers for mitochondria.

Key words: *Spirodela 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 (plasma-lemma) 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)

and low sensitivity to azide and oligomycin (Stout and Cleland 1982, O'Neill et al. 1983, Bennett et al. 1984). Recently it has been reported that vanadate and NO_3^- selectively inhibit the plant plasma membrane (Bowman and Slayman 1979, Cocucci et al. 1980, DuPont et al. 1981, O'Neill and Spanswick 1984) and tonoplast ATPase respectively (Admon et al. 1981, Walker and Leig 1981, O'Neill et al. 1983, Bennett et al. 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) *Spirodela 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 $13000 \times g$ for 10 min. The resulting supernatant was centrifuged at $80000 \times g$ for 30 min. The $80000 \times g$ 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 $80000 \times g$ 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 repelleted at $8000 \times g$ for 30 min. Pellets were resuspended in 250 mM sucrose, 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 cm^3 reaction mixture containing 33 mM Tris/Mes buffer (pH 6.5), 3 mM ATP-Tris, 2.5 mM MgSO_4 , 50 mM KCl and 25 mM K_2SO_4 . For assaying ATPase activity in the presence of NO_3^- , 50 mM KNO_3 was added instead of 25 mM K_2SO_4 . 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 KNO_3 . ATPase activity in the presence of KNO_3 was lower than in its absence, which may indicate that there

Fig. 1. The effect of NO_3^- on inhibition of ATPase activity by vanadate. Activity was assayed in the presence of 3 mM ATP, 2.5 mM MgSO_4 , 50 mM KCl, 25 mM K_2SO_4 , 33 mM Tris/Mes (pH 6.5) and 0.2 cm^3 of crude membrane fraction (about 245 μg protein) and in the presence (●) or absence (○) of 50 mM KNO_3 , with NO_3^- -sensitive ATPase (■) as the difference between the two. When KNO_3 was used, K_2SO_4 was omitted

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 KNO_3 . However, it seems that the degree of inhibition by NO_3^- (ΔNO_3^-) did not change as the result of increasing vanadate concentration. This may indicate that vanadate and NO_3^- inhibit different enzymes.

Total ATPase activity distribution on a continuous sucrose density gradient

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 MgSO_4 , 25 mM K_2SO_4 and 50 mM KCl. The total activity of NO_3^- -sensitive ATPase (ΔNO_3^-) (B) was calculated as the difference between the total activity and that determined in the presence of 50 mM KNO_3 substituted for 25 mM K_2SO_4 in the reaction medium. The azide-sensitive ATPase activity (ΔN_3^-) (B) was calculated as the difference between the total activity and that determined in the same medium with the addition of 2.5 mM NaN_3 . The activity of NO_3^- -sensitive ATPase assayed in the presence of 2.5 mM NaN_3 ($\Delta[\text{NO}_3^- + \text{N}_3^-]$) (C) was calculated as the difference between the activity measured in the presence of KNO_3 with or without the addition of N_3^- . The activity of Cyt.c oxidase and sucrose percentage are also presents (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 N_3^- -sensitive (ΔN_3^-) ATPase

Fig. 3. Distribution of ATPase activity on discontinuous sucrose density gradient. Total (A). ΔNO_3^- and ΔN_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_3VO_4 . Cyt.c oxidase is also presented (A)

(Fig. 2B). NO_3^- -sensitive ATPase (ΔNO_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

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

ATPase showed two peak densities, one at $1.09 \text{ g}\cdot\text{cm}^{-3}$ and the other at $1.11 \text{ g}\cdot\text{cm}^{-3}$, 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_3^- (Fig. 3C, $\Delta[\text{NO}_3^- + \text{N}_3^-]\text{ATPase}$). 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% (w/w) sucrose ($1.095\text{--}1.117 \text{ g}\cdot\text{cm}^{-3}$) or vanadate-sensitive 30-35% (w/w) sucrose ($1.127\text{--}1.151 \text{ g}\cdot\text{cm}^{-3}$) 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% (w/w) sucrose) showed a sharper pH optimum than that of the NO_3^- -sensitive enzyme (23-25% (w/w) sucrose).

Table 1

Effect of inhibitors on NO_3^- -and vanadate-sensitive ATPase

Treatment	ATPase activity, $\mu\text{mol P}_i\cdot\text{mg}^{-1}\text{ protein}\cdot\text{h}^{-1}$			
	NO_3^- -sensitive ATPase activity of 23/28% fraction		vanadate-sensitive ATPase activity of 30/35% fraction	
	activity	%	activity	%
Control	4.7	100	7.5	100
DCCD, 100 μM	1.2	25	2.1	28
EDAC, 1 mM	4.3	92	5.8	77
Oligomycin, 5 $\mu\text{g}\cdot\text{cm}^{-3}$	4.6	98	7.7	103
Oligomycin, 10 $\mu\text{g}\cdot\text{cm}^{-3}$	4.4	93	7.3	96
NaN_3 , 1 mM	4.5	96	7.2	96
Na_3VO_4 , 20 μM	4.3	92	1.9	25
KNO_3 , 50 mM	1.4	30	6.8	91

ATPase activity was assayed in the presence of 3 mM ATP, 2.5 mM MgSO_4 , 50 mM KCl, 25 mM K_2SO_4 , 33 mM Tris/Mes buffer (pH 6.5) and 23/28% membrane fraction (about 36 μg protein) or 30/35% membrane fraction (about 38 μg protein). When KNO_3 was used K_2SO_4 was omitted.

Table 1 shows the effect of several inhibitors of ATPase activity associated with membranes collected from either 23-28% (w/w) or 30-35% (w/w) sucrose fractions. With respect to inhibitor sensitivities, both ATPases were highly sensitive to 100 μM DCCD and showed a marked differential sensitivity to vanadate and NO_3^- . The low density ATPase was inhibited by 70% by 50 mM KNO_3 and was weakly sensitive to 20 μM Na_3VO_4 . In contrast, the high density ATPase was inhibited by 75% by 20 μM Na_3VO_4 and was almost insensitive to KNO_3 . Both ATPases were insensitive

to 1 mM azide and 5-10 $\mu\text{g}\cdot\text{cm}^{-3}$ 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 NO_3^- -sensitive, was enriched in low density membranes and was found between 1.095-1.117 $\text{g}\cdot\text{cm}^{-3}$ (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 $\text{g}\cdot\text{cm}^{-3}$ (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 NO_3^- -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 NO_3^- -sensitive ATPase from corn roots.

On the other hand, the two ATPases from *S. polyrrhiza* showed a marked differential sensitivity to NO_3^- and vanadate. The fact that the activity of ATPase associated with low density membranes was highly NO_3^- -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 *Kalanchoë 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 inhibition 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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Lokalizacja, w gradience gęstości, ATPazy wrażliwej na wanad i NO_3^- wyodrębnionej z sterylnych kultur Spirodela polyrrhiza

Streszczenie

W pracy przedstawiono próby rozdzielania i charakterystyki ATPazy związanych z błonami wypreparowanych z sterylnych kultur *S. polyrrhiza*. ATPazy rozdzielono w gradience gęstości sacharozy i rozrózniono na podstawie różnic gęstości błon oraz ich wrażliwości na niektóre inhibitory. Wyniki wskazują, że aktywność ATPazy wrażliwej na NO_3^- , związanej z tonoplastem występowała w gęstości sacharozy między $1.095\text{--}1.117\text{ g}\cdot\text{cm}^{-3}$. Aktywność ATPazy wrażliwej na wanad, związanej z plazmalemą, występowała w gęstości sacharozy między $1.127\text{--}1.151\text{ g}\cdot\text{cm}^{-3}$. Obydwie ATPazy były niewrażliwe na azydek i oligomycynę i były oddzielone od obszaru występowania markera mitochondriów.