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The localization of vanadium- and nitrate-sensitive ATPases in *Cucumis sativus* L. root cells

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Abstract

Distinct separation of plasma membrane and tonoplast membranes was attained by centrifugation of cucumber root microsomes in a sucrose density gradient. The fractions enriched in plasma membranes, identified on the basis of the sensitivity of ATPases to VO_4^{3-}, sedimented at a specific density of 1.1463-1.1513 $g \times cm^{-3}$. They did not exhibit cytochrome oxidase activity and there was only trace activity of the azide-sensitive ATPase in these fractions. The fractions enriched in tonoplast membranes, having peak activity of nitrate-sensitive ATPase, were found in the region of specific densities of 1.1082-1.1175. The presence of vanadium-sensitive and azide-sensitive ATPases was not found in these fractions. The ATPase inhibitors, DCCD, DES and EDAC, inhibited the activity of both vanadium-sensitive and nitrate-sensitive ATPases.

Key words: Cucumis sativus, ATPases, plasma membrane, tonoplast

INTRODUCTION

Recent studies (Sze 1984) have shown that one of the basic criteria making it possible to distinguish among the membrane-associated ATPases in plant cells is their sensitivity to anions such as VO_4^{3-}, NO_3^- and N_3^-. The plasma membrane-associated ATPase is not not sensitive to NO^{-3} and is specifically inhibited by ortho-vanadate (Bowman et al. 1979, Gallagher and Leonard 1982, O'Neill et al. 1983, Lew and Spanswick 1984, Buczek and Sulej 1986), while the tonoplast-associated ATPase is not sensitive to ortho-vanadate, showing at the same time, a very high sensitivity to NO_3^- ions (Admon et al. 1981, Walker and Leigh 1981, Churchill and Sze 1983, Bennett et al. 1984, Buczek and Sulej 1986). Both ATPases have a low sensitivity to azides and oligomycin (Stout and Cleland 1982, O'Neill et al. 1983, Bennett et al. 1984, Buczek and Sulej 1986), in contrast to mitochondrial ATPase, which is inhibited both azides and oligomycin (Grubmeyer and Spencer 1980, Goffeau and Slayman 1981, Bennett et al. 1984).

The effect of other inhibitors on the activity of membrane-associated ATPases is little specific. DCCD – a non-specific inhibitor of plant membrane-associated ATPases (Schoner and Schmidt 1969, Solioz 1984) – inhibits the activity of the ATPases associated with the plasma membrane or tonoplast (O'Neill et al. 1983, Poole et al. 1984, Bennett et al. 1984, Buczek and Sulej 1986). Walker and Leigh (1981) and Buczek and Sulej (1986) have shown that the plasma membrane-associated ATPase was inhibited by the water-soluble carbodiimide (EDAC), while this inhibitor had no effect on the tonoplast ATPase. However, Lin et al. (1977), Bennett and Spanswick (1983), O'Neill et al. (1983) and Poole et al. (1984) observed the inhibition of tonoplast ATPase by EDAC. Similarly, DES inhibited both the activity of the plasma membrane-associated ATP-ase (Bowman et al. 1979, Goffeau and Slayman 1981), and the tonoplast-associated ATPase (Aoki and Nishida 1984, Poole et al. 1984).

Earlier studies conducted in our laboratory (Buczek 1973) showed that sodium metavanadate inhibited the activity of nitrate reductase in tomato leaves. Orthovanadate acted simlarly in experiments on cucumber leaves and roots (Buczek, personal communication). It was also found (Buczek 1980) that vanadium and DCCD inhibited both the activity of nitrate reductase and the activity of ATPases in cucumber and maize roots and clearly reduced the uptake of NO_3^-. It seems probable, then, that there is an interrelationship between the functioning of ATPases and the reduction of nitrates (Butz and Jackson 1977), at least in respect to the active transport of NO_3^- into cells.

Because of the above relationships, studies on the subcellular localization of NO_3^- or VO_4^{3-}-sensitive ATPases and the effects of some ATPase and nitrate reductase inhibitors on the membrane-associated cucumber root ATPases were conducted in the first stage of this study.

MATERIAL AND METHODS

Plant material. The experiments were conducted on 6-day-old cucumber (*Cucumis sativus* L. var. Wisconsin) roots. Cucumber plants which had been germinated in the dark (2 days at 27°C), were grown for 3 days in a nitrogen-free medium of the following composition, in mM: K_2SO_4 – 1; Ca $(H_2PO_4)_2$

– 0.33; $CaSO_4 \times 2\ H_2O$ – 0.7; $MgSO_4 \times 7\ H_2O$ – 0.7. Next, the plants were transferred to a medium with the following composition (mM): KNO_3 – 1.7; Ca $(NO_3)_2$ – 1.7; KH_2PO_4 – 0.33; $MgSO_4 \times 7\ H_2O$ – O.7. Both mediums contained microelements in the following amounts (μM): iron citrate – 75, $MnSO_4$ – 10, $CuSO_4 \times 5\ H_2O$ – 1, $ZnSO_4 \times H_2O$ – 0.01, $NaMoO_4$ – 0. 001, and their pH equalled 6.5. The cucumbers were grown under a 16h photoperiod (10.3 W m^{-2}) and a daytime temperature of 25°C, and nighttime temperature of 22°C.

Isolation of membrane fractions. Approximately 50 g of 6-day-old cucumber roots were ground in a mortar with 200 cm^3 25mM Tris-HCl, pH 7.5, containing 250 mM sucrose, 3 mM EDTA, 4 mM dithiothreitol and 0.1% bovine serum albumin (BSA). The homogenate was filtered through a double layer of Miracloth and centrifuged for 10 min at 13 000 × g. The sediment was discarded and the supernatant was centrifuged for 30 min at 80 000 × g. The pellet was suspended in 2 cm^3 of extraction buffer and applied to either a linear or discontinuous sucrose density gradient. The linear gradient was formed in 16 cm^3 15-45% (w/w) sucrose. The discontinuous sucrose density gradient was made using 20%, 28%, 32% and 42% (w/w) sucrose solutions (4 cm^3 of each). Sucrose was dissolved in 25 mM Tris-MES, pH 7.5, 3 mM EDTA and 4 mM dithiothreitol. The samples were centrifuged for 3h at 80 000 × g. The fraction volume was 1.8 cm^3. The entire procedure was carried out at 0-4°C.

Assay of enzymic activity. ATPase activity was assayed in a reaction medium containing 33 mM Tris-Mes (pH 7.5), 3 mM ATP, 2.5 mM $MgSO_4$, 50 mM KCl and other substances, the type and amounts of which are given in the descriptions of the appropriate figures or tables. After 30 minutes of incubations at 37°C, the reaction was stopped by adding trichloroacteic acid to a final concentration of 5%. The activity of the ATPase was measured by the amount of phosphorous released during the enzymic reaction, as determined by the method of Ames (1966). The cytochrome oxidase activity was assayed according to Smith (1955), determining the rate of oxidation of reduced cytochrome c. The sucrose concentration in each fraction was determined refractometrically.

Abbreviations. VO_4-ATPase – vanadium-sensitive ATPase, NO_3-ATPase – nitrate-sensitive ATPase, N_3-ATPase – azide-sensitive ATPase, DCCD – N-N'-dicyclohexylcarbodiimide, DES – diethylstibestrol, EDAC – 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

RESULTS

Figure 1 presents the separation of cell membranes (pellet obtained at 80 000 × g) from cucumber roots in a linear sucrose density gradient. The particular type of membrane was identified on the basis of the sensitivity of the various membrane-associated ATPases to inhibitors such as vanadium, nitrates and azides. The plasma membrane marker was the VO_4^{3-}-sensitive ATPase, the ATPase inhibited by NO_3^- was used as the tonoplast membrane marker and the ATPase sensitive to azides as the marker for mitochondrial membranes. It was shown that in all of the fractions obtained through centrifugation in a linear sucrose density gradient, there was only trace activity of cytochrome c oxidase, a marker of mitochondria, and that this was limited only to the first fraction with a specific density of 1. 1816 $g \times cm^{-3}$ (Fig. 1b). Peak activity of the vanadium-sensitive ATPase was found in two fractions (3 and 4) with a specific density of 1.1463 and 1.1513. Slight activity of N_3--ATPase was also observed in these fractions. The highest activity of nitrate-sensitive ATPase was seen in fractions 5 and 6, having a specific density of 1.1082 and 1.1175$g \times cm^{-3}$ (Fig. 1a).

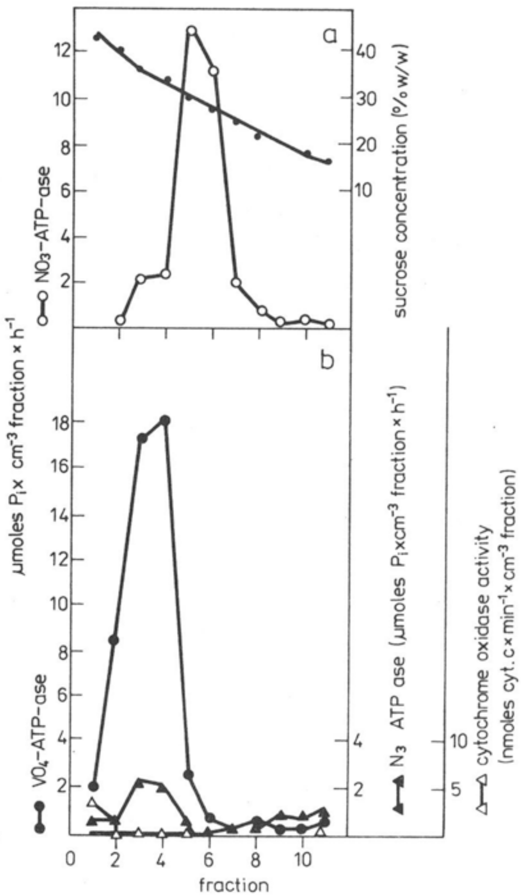

Fig. 1. The distribution of enzymic activity in fractions obtained by centrifugation of *Cucumis sativus* root cell membrane preparations in a linear sucrose density gradient. The activity of ATPases was assayed at pH 7.0. Control samples contained 33 mM Tris-MES, 3mM ATP, 2.5. mM $MgSO_4$, 50 mM KCl, 1mM NaN_3. 50 µM Na_3VO_4 and 50 mM KNO_3. The activity of nitrate- sensitive ATPase (NO_3-ATPase) was assayed without KNO_3, vanadium-sensitive ATPase (VO_4-ATPase) without Na_3VO_4, and of the ATPase inhibited by azides, without NaN_3

Centrifugation of microsomes obtained from cucumber roots in a discontinuous sucrose density gradient made it possible to obtain much better separation of the plasma membranes and tonoplast membranes (Fig. 2). Sharp and distinct separation of the peak activities of vanadium-sensitive and nitrate-sensitive ATPases was obtained. The activity of the VO_4-ATPase was limited to fractions 3 and 4, while the peak activity of NO_3-ATPase (Fig. 2a) was not found until fractions 7 and 8. There was only trace activity of cytochrome oxidase and this was limited to the first fraction. In fractions 1 and 4 a relatively low level of N_3-ATPase was found (Fig. 2b).

Table 1 presents the sensitivity to various inhibitors of the ATPase in the plasma membrane-enriched fractions (specific density of 1.1463-1.1513 $g \times cm^{-3}$) or tonoplast-enriched fractions (specific density 1.1082-1.1175 $g \times cm^{-3}$). It was shown that the ATPases associated with high density membranes, strongly inhibited by vanadium, were not sensitive to nitrates, and that azides only

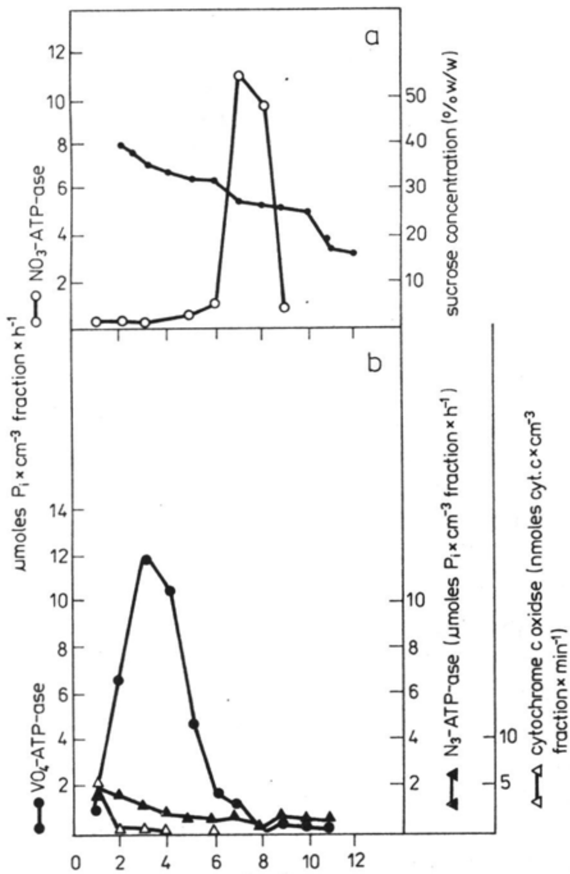

Fig. 2. The distribution of enzymic activity in fractions obtained by centrifugation of a preparation of cell membranes from *Cucumis sativus* roots in a step sucrose density gradient. Details as in Fig.

slightly lowered their activity (by about 25%). However, nitrates strongly inhibited the activity of ATPases in fractions with a low specific density. Vanadium and azide did not have this sort of effect on these ATPases. Both of these ATPases did not differ in their sensitivities to the remaining tested inhibitors. DCCD at a concentration of 20 µmoles significantly lowered the activity of both VO_4-ATPase and NO_3-ATPase. Also DES at a concentration of 100 µmoles clearly inhibited the activity of both ATPases. EDAC used at a concentration of 100 µmoles did not change the activity of both enzymes. This occurred only when a 10-fold greater concentration was used.

Table 1

The effect of inhibitors on the activity of VO_4-ATPase and NO_3-ATPase

Inhibitor (concentration)	µmoles $P_i \times cm^{-3}$ fraction $\times h^{-1}$	
	VO_4-ATPase (fractions 3+4)	NO_3-ATPase (fractions 7+8)
Control*	35.56	24.77
NaN_3 (1 mM)	30.10	23.98
Na_3VO_4 (50 µM)	5.83	23.02
KNO_3 (50 mM)	34.81	4.52
DCCD (20 µM)	14.09	11.05
DES (100 µM)	11.43	9.11
EDAC (100 µM)	37.06	24.06
EDAC (1 mM)	26.29	13.77

* Control samples contained 25 mM Tris-MES pH 7.0, 3 mM ATP, 25 mM $MgSO_4$ and 50 mM KCl.

DISCUSSION

Centrifugation of a preparation of membranes from *Cucumis sativus* root cells in a sucrose density gradient enabled the separation of two different membrane fractions. The first, with a specific density of 1.1463-1.1513 $g \times cm^{-3}$, was characterized by high activity of VO_4-inhibited ATPase. Both the specific density of the fractions and the presence of VO_4-ATPase suggest that it was enriched in plasma membrane. Similar properties were exhibited by plasma membrane preparations isolated from soya (Lew and Spanswick 1984), beets (Poole et al. 1984) and *Spirodella polyrrhiza* (Buczek and Sulej 1986). The peak activity of NO_3-ATPase, taken to be an enzyme specifically associated with tonoplast membranes (Admon et al. 1981, Leigh et al. 1979, Leigh and Walker 1980), was found in fractions with a specific density of 1.1175-1.1082, which is in agreement with preparations obtained from other plants (Bennett et al. 1984, DuPont et al. 1982). Since the VO_4-ATPase did not change its activity when nitrates were added to the incubation medium, and the NO_3-ATPase did not exhibit sensitivity to vanadium (Table 1), it can be accepted that the plasma membrane-enriched fractions obtained as the result of centrifugation of the 80 000×g pellet in a discontinuous sucrose density gradient were free from tonoplast contaminants, and vice versa. The plasma membrane and tonoplast preparations were also free from contamination by mitochondrial membranes, which is indicated by the finding of only trace activities of cytochrome oxidase and N_3-ATPase, or their total lack.

Inhibitors such as DCCD, DES and EDAC did not act specifically on only one membrane-associated cucumber root ATPase. DCCD and DES, thought to be by some authors (Leonard and Hodges 1973, Bowman et al. 1979, Goffeau and Slayman 1981) a specific inhibitor of membrane-associated ATPase, inibited in our experiments both the activity of VO_4-ATPase and NO_3-ATPase. Similar effects of these inhibitors on plasma membrane-associated or tonoplast-associated ATPases of beet cells were observed by Bennett et al. (1984). Also EDAC, which selectively inhibited tonoplast ATPase while not affecting the activity of plasma membrane ATPase in membrane preparations from *Spirodella polyrrhiza* (Buczek and Sulej 1986), in our experiments slightly inhibited the activity of both enzymes.

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Lokalizacja wrażliwych na wanad i azotany ATPaz w komórkach korzeni Cucumis sativus L.

Streszczenie

W wyniku wirowania frakcji mikrosomalnej komórek korzeni ogórka w gradiencie gęstości sacharozy uzyskano wyraźne rozdzielenie błon plazmalemmy i tonoplastu. Frakcje wzbogacone w plazmalemmę, identyfikowane na podstawie wrażliwości ATPaz na VO_4^{3-}, osiadały przy

gęstości właściwej 1,1463-1,1513 g × cm^{-3}. Nie wykazywały aktywności oksydazy cytochromowej, a aktywność ATPazy wrażliwej na działanie azydku była w tych frakcjach jedynie śladowa. Frakcje wzbogacone w tonoplast, wykazujące maksymalną aktywność ATPazy hamowanej przez azotany, znajdowano w obszarze gęstości właściwej 1,1082-1,1175 g × cm^{-3}. W tych frakcjach nie stwierdzono obecności ATPaz wrażliwych na wanad ani ATPaz hamowanych przez azydek. Inhibitory ATPaz (DCCD, DES i EDAC) hamowały aktywność ATPaz wrażliwych na wanad oraz ATPaz wrażliwych na azotany.