Two distinct proton translocating ATPases are present in membrane vesicles from radish seedlings

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Two distinct systems of Mg-ATP-dependent transport of protons have been put in evidence in microsomal vesicles from radish seedlings by investigating the effects of vanadate and of NO_3^- on the initial rate of acridine orange accumulation. One system is vanadate-resistant and is inhibited by NO_3^- (I_{50} 1.3 mM), the other is inhibited by vanadate (I_{50} 43 μM) and unaffected by NO_3^-. The two systems differently distribute along a continuous sucrose density gradient: the NO_3^--sensitive system of Mg-ATP-dependent transport of protons peaks at d = 1.13–1.14, while the vanadate-sensitive one peaks at d = 1.16–1.17.

Membrane vesicle *Radish* *Proton translocating ATPase* *Vanadate-sensitive ΔpH* *NO_3^--sensitive ΔpH* *Acridine orange*

1. INTRODUCTION

Work on membrane ATPases from plant materials led to identification of besides the well-known ATPases of mitochondria and chloroplasts, two main types of Mg^{2+}-dependent ATPases; localized at the tonoplast and at the plasma membrane, respectively. These two ATPases can be distinguished, besides by their localization, on the basis of two biochemical characteristics:

(i) Plasma membrane ATPase is inhibited by vanadate coherently with the finding that the purified enzyme forms a phosphorylated intermediate upon addition of Mg-ATP. Inversely, tonoplast ATPase results are unaffected by vanadate;

(ii) Plasma membrane ATPase is activated by alkali cations, K^+ generally being the preferred one, and is insensitive to a variety of anions (Cl^-, SO_4^{2-}, NO_3^-). Inversely, tonoplast ATPase results virtually unaffected by monovalent cations and markedly sensitive to anions; in particular, it is activated by Cl^- (and, to a variable extent, by some organic anions) and strongly inhibited by NO_3^- [1–9].

Both of these enzymes appear involved in the electrogenic transport of protons: plasma membrane ATPase could catalyze proton extrusion to the apoplasmic space, while tonoplast ATPase could catalyze accumulation of protons into the vacuole. Proton transport activity of the plasma membrane ATPase of yeast and fungi has been extensively studied both in native vesicles and in reconstituted liposomes ([3] and references therein); moreover evidence for proton transport activity has also been presented for the plasma membrane ATPase partially purified from oat roots and reconstituted into liposomes [5]. Evidence for ATP-dependent proton transport has been reported also for highly purified vacuoles and tonoplast vesicles [8,10].

As far as higher plants are concerned, most of the work on ATP-dependent proton transport in

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; BisTris, 2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; AO, acridine orange; MES, 2-(*N*-morpholino)ethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoro methoxyphenylhydrazone

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native vesicles has been performed on crude or on dextrane-purified microsomes [11–19], both consisting of a mixed population of endoplasmic reticulum, Golgi, plasmamembrane and tonoplast. ATP-dependent proton accumulation into these vesicles is stimulated by Cl^-; such effect seems to depend not only on the capability of Cl^- to depolarize $\Delta\psi$, but also on a specific activation by Cl^- of a proton-translocating ATPase [11,15,18,19]. Data on the effect of vanadate on ATP-dependent electrogenic transport of protons into these vesicles are conflicting: in some materials no inhibition by vanadate has been observed, in others a partial inhibition by vanadate has been reported [11,13–17,19].

Here, we show that NO_3^- specifically inhibits the vanadate-resistant portion of ATP-dependent proton transport in microsomes from radish seedlings. Differential sensitivity to inhibition by NO_3^- and by vanadate allowed us to demonstrate that 2 distinct ATP-dependent proton pumps, localized on different membranes, co-exist in microsomes from radish seedlings.

2. MATERIALS AND METHODS

2.1. *Plant material*

Radish seeds (*Raphanus sativus* L., c.v. Tondo-Rosso Quarantino, Ingegnoli, Milan) were germinated for 72 h in the dark in daily renewed distilled water, in an agitated (70 spm) bath at 26°C.

2.2. *Preparation of membrane fraction*

Decoated seedlings were ground in a mortar with 4 vol. 0.3 M sucrose, 0.1 mM $MgCl_2$, 1 mM EDTA, 0.35‰ β-mercaptoethanol, 0.5% BSA, 50 mM Tris adjusted to pH 8 with HCl. Membrane fraction sedimenting between 13000 and 80000 × *g* was resuspended in 0.25 M sucrose, 0.1 mM $MgCl_2$, 0.35‰ β-mercaptoethanol, 0.5% BSA, 1 mM Tris adjusted at pH 7 with HCl, and centrifuged for 30 min at 80000 × *g*. Pellet was resuspended with a syringe and a 26 gauge needle in 0.25 M sucrose, 3 mM DTT, 0.2% BSA, 1 mM BisTris–Hepes (pH 7) (resuspension medium) at 1–3 mg protein/ml. Membrane aliquots were stored at −70°C for up to 10–15 days without loss of ATP-dependent proton translocating activity.

2.3. *Density gradient centrifugation*

Microsomes (2 ml at 5 mg protein/ml) were layered over a 20–45% (w/w) sucrose gradient, made in 0.1 mM $MgSO_4$, 0.35‰ β-mercaptoethanol, 0.5% BSA, 1 mM Hepes–Tris (pH 7). Gradients were centrifuged in a Spinco SW 27 rotor at 27000 rev./min for 3 h. Fractions (1.8 ml/tube) were diluted 1:1 with 0.5% BSA plus 0.35‰ β-mercaptoethanol and centrifuged 30 min at 50000 rev./min in a Spinco 50 Ti rotor. Pellets were resuspended with a syringe and a 26 gauge needle in a resuspension medium (0.45 ml/fraction) and stored at −70°C before use.

2.4. *Determination of AO absorbance*

AO concentration was measured as absorbance change at 492 nm with 550 nm as the reference wavelength by a Sigma ZWS II dual wavelength spectrophotometer equipped with a Philips PM8222 recorder. Assays were performed at room temperature (22–25°C). Vesicles (0.05–0.1 ml) were incubated in 2 ml final vol. in the presence of 7.5 μM AO, 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 17.5 mM MES buffered at pH 6.5 with Tris, 5 mM $MgSO_4$ and 50 mM KCl or KNO_3 (unless otherwise specified). The reaction was initiated by addition of 20 μl 0.3 M ATP buffered at pH 6.5 with Tris.

All the experiments were performed at least 3 times with 3 replicates; standard error of the mean did not exceed ± 8% of the reported values.

2.5. *Protein determination*

Microsomal membranes were washed twice with 1 mM $MgSO_4$ and protein content was determined by the Lowry method as modified [20] with BSA as standard.

2.6. *Materials*

AO, vanadium-free ATP disodium salt, essentially fatty acid free BSA (fraction V), DTT, FCCP were obtained from Sigma. Sodium orthovanadate was from Fisher. All other chemicals were analytical grade.

3. RESULTS

3.1. *AO as ΔpH probe*

AO, a weak base which permeates membranes in its unprotonated form, has been extensively used

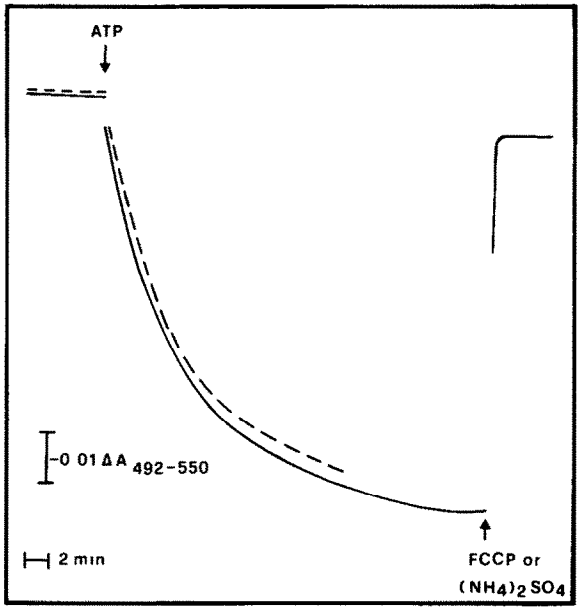

Fig.1. Time course of ATP-induced AO accumulation. Microsomes (0.1 mg protein) were incubated as in section 2, in the presence of 50 mM KCl, in the absence (——) or in the presence (– – –) of 0.5 μg oligomycin/ml. FCCP and $(NH_4)_2SO_4$ were added at a final concentration of 10 μM and 2 mM, respectively.

to monitor inside acid transmembrane ΔpH in membrane vesicles from both animal and plant tissues: acidification of the intracellular space determines an accumulation of AO, measured as decrease of dye absorbance [14,21].

Fig.1 shows the time course of AO uptake (measured as decrease of $\Delta A_{492-550}$) into microsomal vesicles: in the presence of KCl and $MgSO_4$ addition of ATP determines an accumulation of AO: the rate of AO accumulation is linear for 1–2 min, then it slows down and tends to level off only after 30 min. Oligomycin does not significantly affect ATP-induced AO accumulation. Addition of FCCP or $(NH_4)_2SO_4$ determines an immediate release of AO. Moreover no ATP-induced accumulation of AO is observed in membrane vesicles pretreated with the uncouplers (not shown).

These results suggest that ATP-induced accumulation of AO reflects the building up of an inside acid ΔpH mediated by non-mitochondrial Mg^{2+}-dependent proton translocating ATPase(s).

The slow kinetics of ATP-dependent proton translocation (a common feature of proton translocating ATPases in membrane vesicles from higher plants [11,13,17,18]) together with the rapid equilibration of AO (see the rapid efflux of AO upon addition of the uncouplers in fig.1 and [14]) renders it possible to monitor the initial rate of ATP-dependent proton pumping by measuring the initial rate of AO accumulation. In all the following experiments the initial rate of ATP-induced AO accumulation ($\Delta A_{492-550}$/min) was measured in the presence of 0.06–0.12 mg protein/sample and of 7.5 μM AO: these concentrations fell within the range of linearity of the dye response with respect to both protein concentration and AO concentration (not shown).

3.2. *Effect of NO_3^- and of vanadate on ATP-driven proton transport*

Preliminary data on the ATPase activity in the microsomal fraction utilized in these experiments showed that:

(i) The ATPase activity measured in the presence of KCl was higher than that measured in the presence of KNO_3;

(ii) Vanadate at up to 200 μM only partially inhibited the ATPase activity measured in the presence of KCl (M.C. Cocucci, personal communication).

We investigated the effect of NO_3^- and of vanadate on the initial rate of ATP-induced AO accumulation. The rate of ATP-induced AO accumulation is much higher in the presence of KCl than in the presence of KNO_3, both in the absence and in the presence of 60 μM vanadate (table 1).

Table 1

Effect of NO_3^- and of vanadate on ATP-induced AO accumulation

	Control	Vanadate	Inhib. by vanadate
	($-\Delta A_{492-550} \cdot min^{-1} \cdot mg\ protein^{-1}$)		
50 mM KCl	0.140	0.112	0.028
100 mM KCl	0.167	0.138	0.029
50 mM KNO_3	0.042	0.017	0.025
100 mM KNO_3	0.047	0.023	0.024
50 mM KCl + 50 mM KNO_3	0.058	0.028	0.030

Initial rate of ATP-induced AO accumulation was measured in the absence and in the presence of 60 μM vanadate

When 50 mM KNO_3 is supplied together with 50 mM KCl, the rate of ATP-induced AO accumulation is much lower than that measured in the presence of KCl alone and only slightly higher than that measured in the presence of KNO_3 alone. Vanadate inhibits the initial rate of ATP-induced AO accumulation both in the presence of KCl and in the presence of KNO_3; if computed on a percent basis the effect of vanadate results much higher (50–60%) in the presence of KNO_3 than in the presence of KCl alone (about 20%). However, the net effect of vanadate (see last column of table 1) is about the same in the presence of KCl, KNO_3 or of both.

The finding that vanadate-sensitive proton translocating activity is virtually the same in the presence of Cl^- or of NO_3^-, while it is lower in the presence of SO_4^{2-} (not shown) is consistent with the observations that:

(i) Cl^- and NO_3^- have similar permeabilities [22] and thus similar capabilities to collapse the electrical component of $\Delta\bar{\mu}_{H^+}$;

(ii) Vanadate-sensitive ATPase is not specifically activated or inhibited by Cl^- and by NO_3^-, respectively [1,5].

The simplest interpretation of the above results is that ATP-induced AO accumulation measured in the presence of KCl represents the sum of the activities of two distinct ATP-dependent systems of proton translocation: (i) a vanadate-sensitive, anion-insensitive system; and (ii) a vanadate-resistant, NO_3^--sensitive one.

Fig.2 shows the kinetics of inhibition by NO_3^- of the initial rate of ATP-induced AO accumulation in the presence of 50 mM KCl: the inhibition rises with NO_3^- reaching a maximum at about 10 mM KNO_3. The inset to fig.2 represents the plot of the reciprocals of NO_3^--inhibited activity (i.e., the initial rate of ATP-induced AO accumulation measured in the presence of 50 mM KCl minus that measured in the presence of 50 mM KCl plus KNO_3) *vs* the reciprocals of KNO_3 concentrations: the data fit a straight line, thus allowing extrapolation of I_{50} for NO_3^- and of the maximum value of NO_3^--sensitive activity. In the presence of 50 mM KCl I_{50} for NO_3^- results 1.3 mM and about 62% of total activity would be sensitive to inhibition by NO_3^-.

The kinetics of inhibition by vanadate has been studied in the presence of 50 mM KNO_3 to

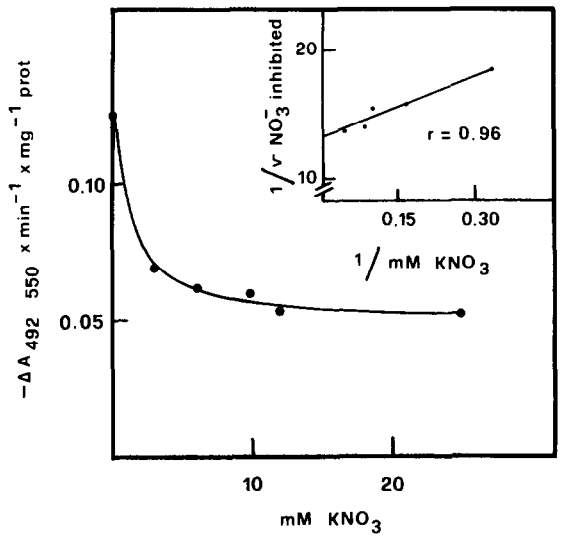

Fig.2. Inhibition by NO_3^- of the initial rate of ATP-induced AO accumulation. Incubation was run in the presence of 50 mM KCl plus KNO_3 at the specified concentrations. Inset: reciprocals of NO_3^--inhibited ativity (i.e., $\Delta A_{492-550}\cdot min^{-1}\cdot mg$ protein^{-1} in the presence of KCl $- \Delta A_{492-550}\cdot min^{-1}\cdot mg$ protein^{-1} in the presence of KCl + KNO_3) are plotted *vs* reciprocals of KNO_3 concentrations.

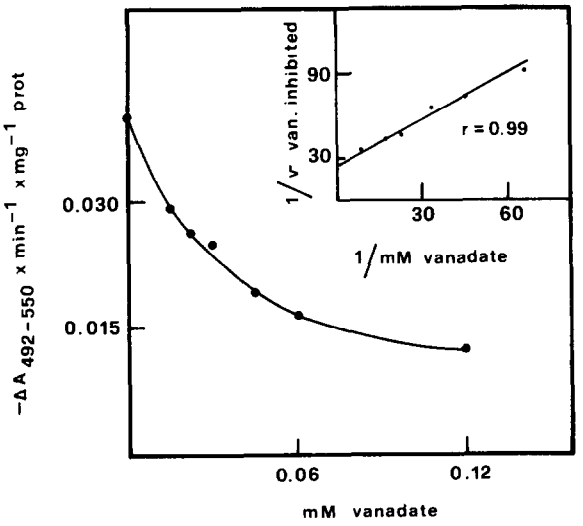

Fig.3. Kinetics of inhibition by vanadate of the initial rate of ATP-induced AO accumulation. Incubation was run in the presence of 50 mM KNO_3. Inset: reciprocals of vanadate-inhibited activity (i.e., $\Delta A_{492-550}\cdot min^{-1}\cdot mg$ protein^{-1} in the presence of $KNO_3 - \Delta A_{492-550}\cdot min^{-1}\cdot mg$ protein^{-1} in the presence of KNO_3 + vanadate) are plotted *vs* reciprocals of vanadate concentrations.

minimize the vanadate-resistant component of ATP-induced AO accumulation. The results reported in fig.3 show that the inhibition by vanadate increases with vanadate concentration and does not reach saturation within the concentration range tested. The plot of reciprocals of vanadate-inhibited activity *vs* reciprocals of vanadate concentrations (inset to fig.3) gives an I_{50} for vanadate of 43 μM and indicates that all of the activity measured in the presence of KNO_3 would be sensitive to inhibition by vanadate. It is worth to note that I_{50} for vanadate has been obtained by measurements of inhibition by vanadate of the initial rate of ATP-induced AO accumulation and that the effect of vanadate increases during incubation in the presence of ATP, reaching a maximum only after 3–5 min (not shown).

3.3. *Distribution of ATP-driven proton transport on sucrose gradient*

Fig.4 shows the distribution along a continuous sucrose gradient of ATP-induced AO accumulation measured in the presence of KCl plus or minus vanadate or in the presence of KNO_3. All the activities broadly distribute along the gradient: however the activity measured in the presence of KCl plus vanadate peaks in a lighter region of the gradient (d = 1.13–1.14) than that measured in the presence of KNO_3, which peaks at d = 1.16–1.17.

Vanadate-sensitive activity (computed as the difference between values measured in the presence of KCl and those measured in the presence of KCl plus 60 μM vanadate) comigrates with the activity measured in the presence of KNO_3 towards the denser zone of the gradient. NO_3^--sensitive activity (computed as the difference between the values measured in the presence of KCl and those measured in the presence of KNO_3) peaks at the same density as that measured in the presence of KCl plus vanadate.

These data are fully consistent with the previous conclusion that ATP-induced AO accumulation measured in the presence of KCl reflects the activities of two distinct systems of ATP-dependent proton translocation and indicate that they are localized in membranes of different densities. One system (localized in low density membranes) is insensitive to vanadate and blocked by NO_3^-, the other (localized in high density membranes) is insensitive to NO_3^- and inhibited by vanadate. The

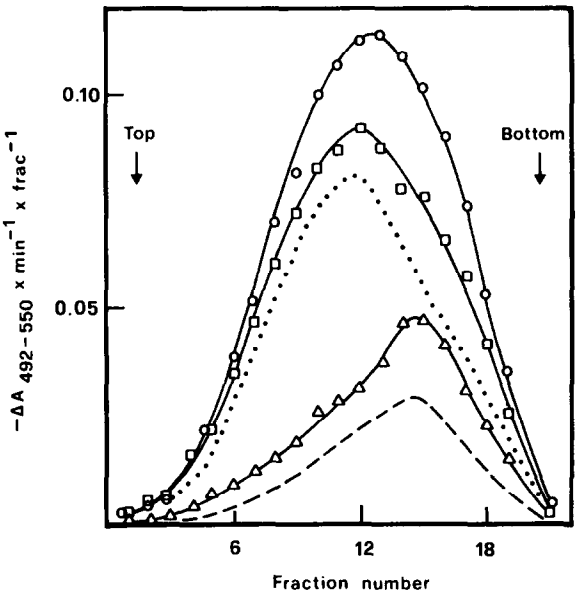

Fig.4. Distribution of ATP-induced AO accumulation along a continuous (20–45%, w/w) sucrose gradient. Initial rate of ATP-induced AO accumulation was measured in the presence of: 50 mM KCl (○), 50 mM KCl plus 60 μM vanadate (□), 50 mM KNO_3 (△). NO_3^--sensitive activity (···) was computed as the difference between activity in the presence of KCl and activity in the presence of KNO_3; vanadate-sensitive activity (– – –) was computed as the difference between activity in the presence of KCl and activity in the presence of KCl plus vanadate.

lower values computed for NO_3^--sensitive and for vanadate-sensitive activity as compared to those obtained for vanadate-resistant and for NO_3^--resistant activity, respectively, depend on the fact that vanadate at the concentration used (60 μM) inhibits only about 60% of the vanadate-sensitive activity (see fig.3).

4. CONCLUSIONS

We have shown two distinct systems of ATP-dependent proton translocation in microsomal vesicles from radish seedlings, on the basis of their different sensitivities to inhibition by NO_3^- and by vanadate: one system is inhibited by vanadate and insensitive to NO_3^-, while the other is inhibited by NO_3^- and insensitive to vanadate. The two systems result localized in membranes characterized by different densities: namely the NO_3^--resistant,

vanadate-sensitive one peaks at d = 1.16–1.17, while the other peaks at lower density (d = 1.13–1.14).

On the basis of their differential sensitivity to NO_3^- and to vanadate, and of their distribution along the gradient, the two systems can be tentatively localized at the plasma membrane (the vanadate-sensitive system) and at the tonoplast (the NO_3^--sensitive system).

These results are consistent with the observation that the microsomal membranes utilized in this work contain at least two ATPases: one, recently purified by M.C. Cocucci (personal communication), presents the characteristics of a plasma membrane ATPase (K^+-stimulated, insensitive to anions, vanadate-sensitive and forming an ATP-dependent phosphorylated intermediate); the other presents some characteristics of tonoplast ATPase (vanadate-insensitive, NO_3^--inhibited, Cl^--stimulated).

Vanadate-sensitive proton transport is a minor portion of total ATP-dependent proton transport measured in microsomal vesicles from radish seedlings (about 30%) and it is not possible to obtain from sucrose density gradients a fraction enriched in vanadate-sensitive ATP-dependent transport of protons reasonably free from contamination by the vanadate-resistant one. Our finding that NO_3^- is able to completely inhibit vanadate-resistant ATP-dependent transport of protons without affecting the vanadate-sensitive system provides a good tool to study the activity of the vanadate-sensitive system in membrane fractions of mixed composition.

In our experiments, proton transport can be put in evidence only in sealed vesicles which present their cytoplasmic face to the medium and pump protons into the intravesicular space. Since the percentage of sealed and well-oriented vesicles can vary for vesicles of different membrane origin, the observed ratio between vanadate-sensitive and NO_3^--sensitive ATP-dependent transport of protons does not necessarily reflect the ratio between vanadate-sensitive and NO_3^--sensitive ATPase activities.

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