# $H^+$/ion antiport as the principal mechanism of transport systems in the vacuolar membrane of the yeast *Saccharomyces carlsbergensis*

L.A. Okorokov, T.V. Kulakovskaya, L.P. Lichko and E.V. Polorotova

*Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences, Pushchino, Moscow Region 142292, USSR*

Received 26 September 1985

The secondary transport systems of the yeast vacuolar membrane have been investigated by (i) the method of radioactive isotopes ([$^{14}$C]arginine); (ii) activation of $H^+$-ATPase by cations ($Cat^+$), when the enzyme is under $H^+$ control and (iii) measurement of changes in the proton gradient ($\Delta$pH) and membrane potential ($E_m$) due to the supposed substrates of the transporters. The main mechanism of cation transport across the yeast tonoplast is probably $H^+/Cat^+$ antiport. The apparent $K_m$ of antiporters for $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$ and $P_i$ are 0.06, 0.3, 0.8, 0.055–0.17 and 1.5 mM, respectively.

*$H^+$-ATPase Cation $H^+$ Antiporter Vacuolar membrane (Yeast)*

## 1. INTRODUCTION

Several groups have reported on the localization of basic amino acids, vitamins, derivatives of purine bases [1] and inorganic ions [2] in fungal vacuoles. The existence of the transport systems of basic amino acids (arginine, lysine, etc.) and purine bases in the tonoplast has also been proved [1,3–5].

The presence of a specific ATPase in the vacuolar membrane, other than the ATPases of mitochondria and plasmalemma, was demonstrated by its solubilization, partial purification [6,7] and reconstitution into liposomes [8]. It was also found that yeast vacuolar membranes possessed the ATP-dependent $H^+$ pump [9] identified as $H^+$-ATPase [10] which supported the $\Delta\tilde{\mu}H^+$-dependent transport of arginine [11], $Ca^{2+}$ [12,13] and other ions [10,13].

We present here new evidence of the presence of $H^+$/ion antiporters in the vacuolar membrane of the yeast *Saccharomyces carlsbergensis* operating at the expense of $\Delta\mu H^+$ formed by the tonoplast ATPase.

## 2. MATERIALS AND METHODS

Yeasts were grown, vacuoles isolates, and protein and enzyme activities determined as described [7,8,10].

Measurement of fluorescence quenching of ACMA was described earlier [10], however, the protein content was 30–50 $\mu$g/ml incubation medium. The membrane potential ($E_m$) was qualitatively determined by fluorescence-quenching measurement of the dye oxanol V (4.5 $\mu$M) [10,13]. The incubation medium consisted of 100 mM sorbitol, 10 mM Mes, adjusted with NaOH to pH 7.2 ($\Delta$pH measurement) or pH 6.5 ($E_m$ measurement). Fluorescence of oxanol V was measured in a Hitachi MPF-4 fluorescence spectrophotometer; excitation and emission wavelengths were 580 and 640 nm, respectively.

Arginine transport into isolated vacuoles was demonstrated by the accumulation of [$^{14}$C]-arginine. 20 $\mu$l of 30 mM MgATP, pH 6.5, and 500 $\mu$l vacuolar suspension (270–470 $\mu$g protein) were introduced into conical test-tubes (Eppendorf). The suspension obtained was incubated at room

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/85/$3.30 

temperature (22°C) for 3 min, supplemented with 10–20 $\mu$l [$^{14}$C]arginine up to a concentration of 40–50 $\mu$M and again incubated for 3 min with mixing. After the addition of cold 10 mM Mes-Na buffer and 0.1 M sorbitol (pH 6.5) up to 1 ml, the suspension was shaken and centrifuged (28 000 × $g$) for 5 min at 0°C. The supernatant was removed and the test-tube walls dried with a paper filter. To each pellet, 200 $\mu$l of 2% Triton X-100 were added. 100-$\mu$l samples were dried on Whatman GF/B, and the radioactivity determined in a liquid scintillation spectrometer.

## 3. RESULTS AND DISCUSSION

### 3.1. *Ion activation of $H^+$-ATPase when the enzyme is under $H^+$ control*

$H^+$ translocation catalyzed by ATPase located on the cytoplasmic surface of vacuolar membranes acidified the intravacuolar space. The generation of $\Delta$pH (acid inside) and $E_m$ (positive inside) inhibited the ATPase, indicating that the enzyme was under $H^+$ control [10,13]. Measurement of the activity of the vacuolar $H^+$-ATPase being under $H^+$ control revealed its stimulation by free $Mn^{2+}$ (fig.1). The increase in concentration of free $Mg^{2+}$ or $K^+$ also stimulated ATPase when the substrate was MgATP (not shown). $Mg^{2+}$ activated the ATPase released from the $H^+$ control (measurements with 50 mM Tris-Pipes) [14,15] by no more than 10–15%, while $Mn^{2+}$ even inhibited it. The apparent $K_m$ of the $H^+/M^{n+}$ antiporters determined from the data on ATPase activation

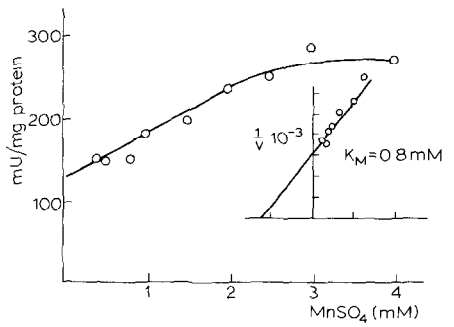


Fig.1. Relation between the specific activity of the ATPase of isolated intact vacuoles and concentration of free $Mn^{2+}$. (Inset) Lineweaver-Burk plot.

were 0.8 and 0.3 mM for $Mn^{2+}$ and $Mg^{2+}$, respectively. ATPase activation by $Zn^{2+}$ and arginine ($Arg^+$) ions allowed for estimation of the apparent $K_m$ of the corresponding antiporters (55 and 7 $\mu$M, respectively).

### 3.2. *$\Delta\tilde{\mu}H^+$-dependent transport of [$^{14}$C]arginine into isolated vacuoles*

Isolated vacuoles of *S. carlsbergensis* retained their radioactivity after incubation with [$^{14}$C]-arginine. The introduction of MgATP into the incubation system increased [$^{14}$C]arginine accumulation 5-fold. Such MgATP-stimuled accumulation of the amino acid was inhibited by DCCD, DES and combination of the protonophore FCCP with valinomycin (table 1). Potassium sulphate inhibited the ATP-stimulated ($\Delta\tilde{\mu}H^+$-dependent) accumulation of [$^{14}$C]arginine by isolated vacuoles by less than 50%. Its effect was hardly different from KSCN, though the permeant anion $SCN^-$ converts $E_m$ to $\Delta$pH [16]. Evidently, arginine transport into vacuoles occurs at the expense of both $E_m$ and $\Delta$pH, and is partially inhibited by $K^+$ competing with arginine for intravacuolar protons.

### 3.3. *Changes in $\Delta$pH and $E_m$ by ions when ATPase is operating or switched off*

It is logical to expect that, when ions are transported into vacuoles at the expense of $\Delta$pH or $E_m$, their flux should change the value of these components of $\Delta\mu H^+$. The changes are easier to observe when the ATPase is switched off or when the rates of $\Delta$pH and $E_m$ creation by the ATPase are lower than those of their consumption. $Ca^{2+}$ added before MgATP decreases the $\Delta$pH (fig.2A) and induces the dissipation of the $H^+$ gradient, being added after the creation of $\Delta$pH (fig.2B). It is noteworthy that the membrane potential increases under such conditions (fig.2C) [13]. Such an effect of $Ca^{2+}$ on $\Delta$pH and $E_m$ agrees well with the idea of the electroneutral $Ca^{2+}/2\ H^+$ antiporter when ATPase is activated by 60% [13]. The effect of $K^+$ and $Zn^{2+}$ on $E_m$ differs from that of $Ca^{2+}$. These ions decrease the formation of $E_m$ when added before MgATP (fig.3). Taking into account that $Zn^{2+}$ decreases both $\Delta$pH (fig.2A) and $E_m$ (fig.3) one can assume that the $Zn^{2+}/H^+$ antiport is electrogenic. According to another interpretation, $Zn^{2+}$ increases the unspecific proton permeability

Table 1

Effect of various reagents on the accumulation of [$^{14}$C]arginine by isolated vacuoles of the yeast *S. carlsbergensis*

| Additions | None | 1.25 mM MgADP | 1.25 mM MgATP | 1.25 mM MgATP | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1% ethanol | 50 $\mu$M DCCD | 50 $\mu$M DES | 1 $\mu$M FCCP + 1 $\mu$M valinomycin | 1 mM cold arginine | 10 mM KSCN | 10 mM $K_2SO_4$ |
| [$^{14}$C]Arginine accumulation* (%) | 100 | 104 | 416 | 360 | 124 | 111 | 117 | 100 | 176 | 200 |

*Mean values of 3 experiments

of the tonoplast and is transported by the electroneutral $Zn^{2+}/2\ H^+$ antiporter.

To evaluate the kinetics of the $H^+$/ion antiporters quantitatively we determined the initial velocities of $\Delta$pH dissipation depending on the ion concentrations. For this purpose we selected conditions for blocking the $H^+$-ATPase when the main part of $\Delta$pH had been created. On addition of DCCD under such conditions, the $\Delta$pH created was not dissipated and did not increase further (fig.4) for more than 10 min. However, the addition of $Ca^{2+}$ or $Zn^{2+}$ results in the dissipation of $\Delta$pH (fig.4). The data on the relation between the initial rate of $\Delta$pH dissipation and $Ca^{2+}$ concentration make it possible to determine the apparent $K_m$ for the $Ca^{2+}/2\ H^+$ antiporter: 60 $\mu$M $Ca^{2+}$. The $K_m$ of the corresponding antiporter for $Zn^{2+}$ thus determined is equal to 170 $\mu$M (vs 55 $\mu$M determined from the enzyme activation) and 1.5 mM for orthophosphate.

The above data agree well with the idea of the availability of $H^+$/ion antiporters in the vacuolar membrane of the yeast *S. carlsbergensis*. The $H^+$/ion antiport is evidently the principal mechanism of transport across the tonoplast. It is used to transfer inorganic cations ($Mn^{2+}$, $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $K^+$), organic cations (arginine, lysine,

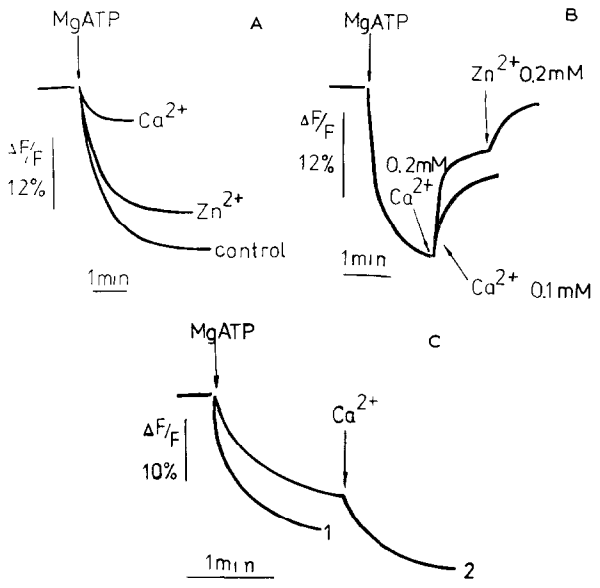


Fig.2. (A) Effect of $Ca^{2+}$ and $Zn^{2+}$ added before MgATP (0.75 mM) on the formation of $\Delta$pH (fluorescence quenching of ACMA). Lower curve, control. $Ca^{2+}$ and $Zn^{2+}$ were added as Mes and sulphate salts accordingly. (B) Effect of $Ca^{2+}$ and $Zn^{2+}$ on the $\Delta$pH created. (C) Effect of $Ca^{2+}$ on the ATPase-dependent formation of $E_m$ (fluorescence quenching of oxanol V). Upper curve, MgATP (0.75 mM), then Ca-Mes (2 mM); lower curve, Ca-Mes (2 mM) and then MgATP (0.75 mM).

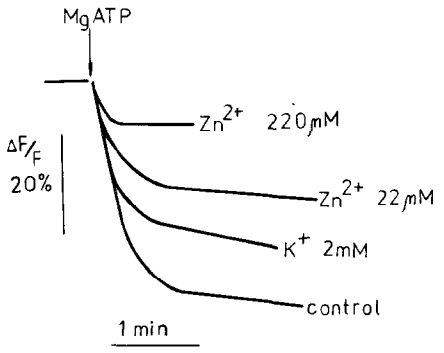


Fig.3. Effect of $Zn^{2+}$ and $K^+$, added before MgATP, upon $E_m$ formation.

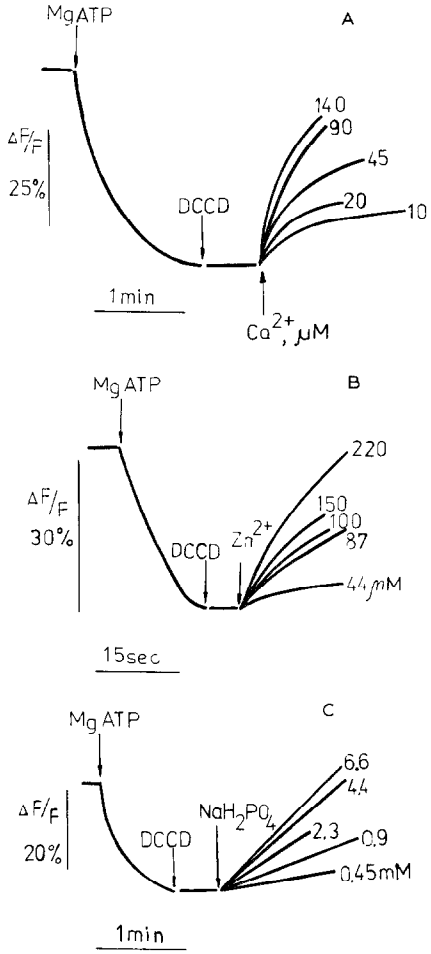


Fig.4. Dissipation of $\Delta$pH by $Ca^{2+}$ (A), $Zn^{2+}$ (B) and phosphate (C) when the vacuolar ATPase is blocked by DCCD (75 $\mu$M). Phosphate added as sodium salt.

etc.) and probably phosphate in the case of $E_m$ dissipation.

The finding of systems for the transport of various ions functioning as antiporters offers new scope for studies of the role of vacuoles and their transport systems in the regulation of the level of ions and some metabolites in the cytosol. This was demonstrated for $K^+$, $Mg^{2+}$, $Mn^{2+}$ and phosphate [17] and proved recently by Nicolay et al. for phosphate [18].

It is noteworthy that the apparent $K_m$ of antiporters for $Mg^{2+}$, $Mn^{2+}$ and phosphate (0.3, 0.8 and 1.5 mM) are sufficiently close to the concentrations of these ions in the cytosol evaluated earlier (1.35, 0.3 and 1 mM, respectively) [2]. One can assume that the transport systems for the above ions operate until the ion levels in the cytosol approach the $K_m$ of these systems. It is also noteworthy that the ATPase of the plasmalemma is inhibited by free $Mg^{2+}$ at 0.4–0.5 mM free $Mg^{2+}$ [19,20], i.e. within the $K_m$ region of the $Mg^{2+}/H^+$ antiporter of the tonoplast and when the vacuolar ATPase is activated by $Mg^{2+}$ (here).

## REFERENCES

[1] Matile, P. (1978) Annu. Rev. Plant Physiol. 29, 193–213.
[2] Okorokov, L.A., Lichko, L.P. and Kulaev, I.S. (1980) J. Bacteriol. 144, 661–665.
[3] Boller, T., Durr, M. and Wiemken, A. (1975) Eur. J. Biochem. 54, 81–91.
[4] Schwencke, J. and De Robinson-Szulmaister, H. (1979) Eur. J. Biochem. 65, 49–60.
[5] Nagy, M. (1979) Biochim. Biophys. Acta 558, 221–232.
[6] Okorokov, L.A., Kulakovskaya, T.V. and Kulaev, I.S. (1981) VIIth International Specialized Symposium on Yeast, Spain, Abstr. p.43.
[7] Okorokov, L.A., Kulakovskaya, T.V. and Kulaev, I.S. (1982) FEBS Lett. 145, 160–162.
[8] Lichko, L.P. and Okorokov, L.A. (1984) FEBS Lett. 177, 233–237.
[9] Kakinuma, Y., Ohsumi, Y. and Anraku, Y. (1981) J. Biol. Chem. 256, 10859–10863.
[10] Okorokov, L.A. and Lichko, L.P. (1983) FEBS Lett. 155, 102–106.
[11] Ohsumi, Y. and Anraku, Y. (1981) J. Biol. Chem. 256, 2079–2082.
[12] Ohsumi, Y. and Anraku, Y. (1983) J. Biol. Chem. 258, 5614–5617.
[13] Okorokov, L.A., Lichko, L.P. and Kulakovskaya, T.V. (1985) in: Biochemistry and Function of Vacuolar ATPase in Fungi and Plants (Marin, B. ed.) pp. 203–211, Springer, Berlin.
[14] Rotenberg, H., Grunwald, T. and Avron, M. (1972) Eur. J. Biochem. 25, 54–64.
[15] Rotenberg, H. and Grunwald, T. (1972) Eur. J. Biochem. 25, 71–74.
[16] Stroobant, P. and Scarborough, G.A. (1979) Proc. Natl. Acad. Sci. USA 76, 3102–3106.
[17] Lichko, L.P., Okorokov, L.A. and Kulaev, I.S. (1982) Arch. Microbiol. 132, 289–293.
[18] Nicolay, K., Scheffers, W.A., Bruinenberg, P.M. and Kaptein, R. (1983) Arch. Microbiol. 134, 270–275.
[19] Ahlers, J., Ahr, E. and Seyfarth, A. (1978) Mol. Cell Biochem. 22, 39–49.
[20] Borst-Pauwels, G.W.F.H. and Peters, P.H.J. (1981) Biochim. Biophys. Acta 642, 173–181.