The identification of a proton pump on vacuoles of the yeast *Saccharomyces carlsbergensis*

ATPase is electrogenic H^+-translocase

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1. INTRODUCTION

Different solutes are accumulated inside the yeast vacuoles [1–3]. A mechanism(s) of the transport energization across tonoplast is unknown. One can believe that tonoplast ATPase could drive the arginine transport and an antiport of Arg^+/H^+ occurs [1–4]. However, the problem of the existence of specific vacuolar ATPase remained unsolved due to the multitude of phosphohydrolases located in vacuoles [5]. The existence of such ATPase was proved by solubilization and purification of the enzyme from alkaline phosphatase, GTPase, UTPase, pyro- and polyphosphatase activities [6,7]. The enzyme activity is insensitive to vanadate and azide which blocked plasmalemma and mitochondrial ATPases accordingly, but is very sensitive to DCCD and DES [7].

Abbreviations: ATPase, adenosine triphosphatase (EC 3.6.1.3); DCCD, dicyclohexylcarbodiimide; DES, diethylstilbestrol; ACMA, 9-amino-6-chloro-2-methoxyacridine; MES, 2-(*N*-morpholino)ethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; PIPES, piperazine-*N*,*N*′-bis-2-ethanesulfonic acid

There is evidence now that vesicles of *S. cerevisiae* tonoplast possess an ATP-dependent electrogenic proton pump [8]. Nevertheless it is still unclear what vacuolar phosphohydrolase is responsible for its activity.

We here describe the ATP-dependent transmembrane proton movement in isolated vacuoles of *S. carlsbergensis* yeast. The results obtained are consistent with the assumption that the electrogenic ATPase of vacuoles is a proton pump.

2. MATERIALS AND METHODS

Isolation of vacuoles and determinations of the protein and the enzyme activities were done essentially as in [7] except that 0.1 M sorbitol was added to the incubation medium for the enzyme determination.

Incubation of vacuoles with the ATPase inhibitors was for $\leqslant$2 min. Ethanol solutions of ionophores and inhibitors were used so that [ethanol] in the incubation systems was $\leqslant$0.5%. Quenching of ACMA fluorescence was measured at 25°C in 2 ml medium containing 0.1 M sorbitol, 10 mM PIPES and 2 mM $MgSO_4$ adjusted to pH indicated with NaOH (figure legends). After addi-

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tion of 50 μl vacuoles (8–16 μg protein) and 100 μl 0.14 mM ACMA, the reaction was started with 50 μl solution containing 0.1 M Na-ATP (Na-GTP, Na-UTP) and 0.1 M $MgSO_4$ adjusted to pH of incubation medium with NaOH. Fluorescence was measured with Hitachi model MPF-4 Fluorescence Spectrophotometer. Excitation and emission wavelengths were 415 nm and 485 nm, respectively.

3. RESULTS

3.1. The H^+-control of vacuolar ATPase

H^+ translocation catalyzed by ATPase located on the outer (cytoplasmic) surface of vacuolar membrane should acidify the intravacuolar space. The generation of H^+-gradient (ΔpH), acid inside, and electrochemical potential ($\Delta\psi$), positive inside, should inhibit the ATPase activity. If the unprotonated form of any weak base is permeant across the tonoplast and the protonated form is much less permeant the base will be accumulated inside vacuoles [10,11]. High concentration of weak base will collapse the H^+ gradient. It will also dissipate if some ionophores increase a relatively low H^+ permeability of the tonoplast. As a result, the weak bases, ionophores and solutes transported antiportly with H^+ would release the H^+-control of vacuolar ATPase (table 1). The low ATPase activity was found in MES–Na^+ buffer when amines or permeant ions were omitted. NH_4Cl, Tris and nigericin in combination with K_2SO_4 (electroneutral exchange K^+/H^+) activated ATPase significantly. The combination of FCCP with valinomycin in the presence of K_2SO_4 was functionally identical to nigericin: ATPase was activated. The activation of ATPase by KSCN was not so high: the permeant anion SCN^- collapsed $\Delta\psi$ and converted it to ΔpH [12]. FCCP and gramicidin D inhibited ATPase; valinomycin, NaN_3, Zn^{2+}, Ca^{2+} and arginine enhanced its activity insignificantly. K_2SO_4 was more effective than KSCN; valinomycin did not increase the K_2SO_4 effect indicating a relatively high potassium permeability of the tonoplast.

The above results hold true for the vacuolar preparations with high ATPase activities (450 nmol P_i.mg protein^{-1}.min^{-1} in MES–Na^+ buffer). The vacuolar preparations with the low ATPase activity (up to 200 nmol P_i.mg protein^{-1}.min^{-1}) exhibited the same properties except for the enzyme activation which was higher. NH_4Cl, KSCN and nigericin in the presence of K_2SO_4, valinomycin and valinomycin with K_2SO_4 stimulated ATPase by a factor of 6.0, 1.3, 2.0, 4.6, 1.9 and 3.3, respectively. FCCP halved the enzyme activity, but FCCP in combination with K_2SO_4 stimulated it 4-times. Arginine (0.5 mM, $ZnSO_4$,

Table 1

Influence of ionophores, ions and arginine on ATPase, GTPase and UTPase activities in isolated vacuoles of *Saccharomyces carlsbergensis* yeast

Medium	Enzyme activity (%)		
	ATPase	UTPase	GTPase
MES–Na, 10 mM (pH 6.5)	100[a]	100[a]	100[a]
Tris–PIPES, 50 mM (pH 6.5)	220	196	143
MES–Na, 10 mM (pH 6.5)			
+ NH_4Cl, 10[c]	273	118	133
+ FCCP, 5[b]	77	61	82
FCCP, 5 + valinomycin, 1 + K_2SO_4, 10	226	82	80
Nigericin, 1 + K_2SO_4, 10	209	84	80
+ KSCN, 10	125	115	108
+ K_2SO_4, 10	149	86	96
Valinomycin, 1	105	102	108
Valinomycin, 1 + K_2SO_4, 10	159	86	102
+ NaN_3, 5	107	129	115
+ $ZnSO_4$, 0.045	104	115	121
Ca-citrate, 0.5	113	61	75
Arginine, 0.5 mM	103	124	124
Gramicidin D, 5	91	118	123

[a] Mean values of the spec. act. of these enzymes were equal to 450, 320 and 210 nmol P_i.mg protein^{-1}.min^{-1}

[b] Ionophore and

[c] Salt concentrations are indicated as μM and mM, respectively

Ca-citrate (0.1–1.0 mM) and K_2SO_4 activated ATPase by a factor of 1.6, 1.4–1.9, 1.6 and 2–3, respectively. The release of H^+ control of ATPase enhanced the enzyme activity up to 1200 nmol P_i.mg protein^{-1}.min^{-1}.

This did not exceed the maximal activity of ATPase of vacuolar preparations with high enzyme activity (up to 1215 nmol P_i.mg protein^{-1}.min^{-1}) when the H^+ control was released.

Many properties of ATPase activity of *S. carlsbergensis* vacuoles (table 1 and [6,7]) are similar to those of *S. cerevisiae* [8]. However, it is activated by Zn^{2+} and K^+ while ATPase of *S. cerevisiae* is half inhibited by 20 μM Zn^{2+} and is not stimulated by K^+ [8]. The K_m of vacuolar ATPase of *S. carlsbergensis* (130 μM, not shown) differs from K_m of the enzyme of *S. cerevisiae* (200 μM) insignificantly, but is much lower than that of plasmalemma ATPases of yeast and fungi: 0.7–3.8 mM [13].

Referring to table 1, GTPase and UTPase are also under H^+ control. ATPase is under a stronger H^+ control than GTPase or UTPase (more effective activation of ATPase by NH_4Cl, Tris, nigericin and SCN^-). Moreover, the results on the influence of different reagents on the phosphohydrolase activities points to a likeness of GTPase and UTPase and their distinction from ATPase (table 1).

3.2. *Fluorescent measurements of proton gradient (ΔpH)*

When ATP was added to the vacuolar suspension, the ACMA fluorescence was quenched indicating acidification of vacuoles (fig.1a). On the addition of the protonophore FCCP to the incubation mixture the fluorescence intensity increased pointing to the dissipation of proton gradient. This protonophore prevented the ATP-dependent ΔpH generation completely (fig.1h) while NaN_3 did it only partially (fig.1f).

At 10 μM, DCCD, the inhibitor of vacuolar ATPase [7], blocked vacuolar acidification by 33%, and 40–60 μM DCCD stopped the proton pump completely (fig.1b,c). The vacuolar acidification was insensitive to oligomycin at $\leqslant$14 μg/mg protein (fig.1e) which inhibited the mitochondrial ATPase [13]. The highest concentrations of oligomycin (333 μg/mg protein) inhibited the ΔpH creation by 65–80% (fig.1d).

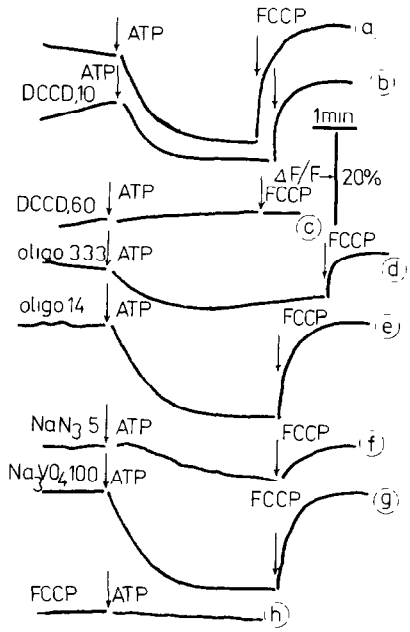

Fig.1. ATP-driven intravacuolar acidification. The influence of DCCD, Na_3VO_4, FCCP (μM), NaN_3 (mM) and oligomycin (μg.mg protein^{-1}) on the ATP-dependent ΔpH formation across the vacuolar membrane.

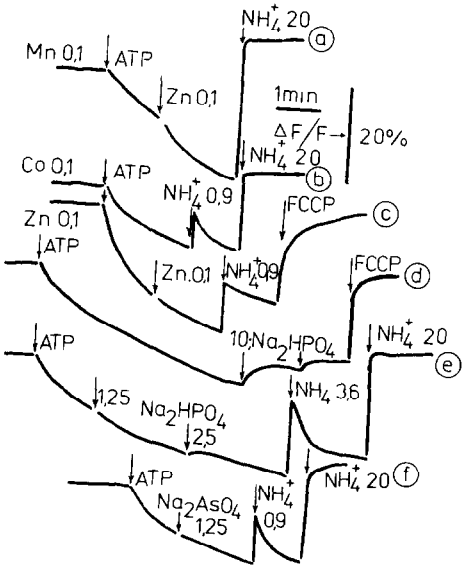

Fig.2. The influence of different cations and anions on the ΔpH generation across the vacuolar membrane. Mn^{2+}, Co^{2+}, Zn^{2+} were added as sulphates in the presence of $MgSO_4$. The final [ion] is indicated as mM and [FCCP] is 5 μM. NH_4^+ were added as NH_4Cl (20 mM).

Vanadate, the potent inhibitor of plasmalemma ATPase of yeast [13], did not affect the vacuolar [7] and mitochondrial [13] ATPases as well as the proton pump of yeast vacuoles (fig.1g).

The omission of Mg^{2+} from the incubation mixture completely prevented the ΔpH generation; Mn^{2+} could substitute Mg^{2+} with nearly the same efficiency (not shown). Mn^{2+} or Co^{2+} (0.1 mM) in the presence of Mg^{2+} did not change the vacuolar acidification; Zn^{2+} (45–90 μM) in the presence of Mg^{2+} stimulated this process (fig.2).

Permeant anion SCN^- collapsing $\Delta\psi$ increased the acidification of vacuoles. This effect was enhanced by increasing the pH (fig.3a–d). I^-, Cl^-, NO_3^- stimulated the ATP-dependent formation of ΔpH in the indicated order (not shown). Inorganic phosphate and arsenate (Na^+ salts, 1.25 mM) did not affect intravacuolar acidification (fig.2e,f); the higher concentrations of phosphate decreased ΔpH (fig.2d,e; second addition). Arginine, 1.1–2.5 mM, stimulated the ΔpH formation and failed to produce this effect in the presence of KSCN (fig.3c–e).

Nigericin + K^+ (1 μM) rapidly collapsed the proton gradient across tonoplast when added after its formation and prevented intravacuolar acidification when added before ATP (fig.3a,b,f). NH_4Cl at 10–20 mM restored the fluorescence response to

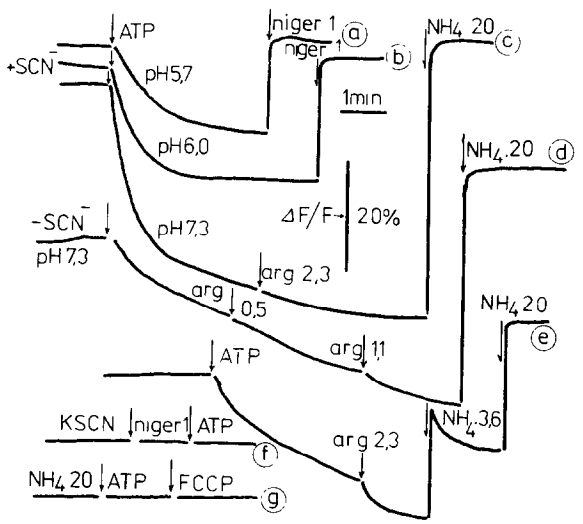

Fig.3. ATP-driven intravacuolar acidification as a function of pH; SCN^-, 10 mM; nigericin, 1 μM; arginine, 0.5–2.3 mM. NH_4^+ were added as NH_4Cl (20 mM).

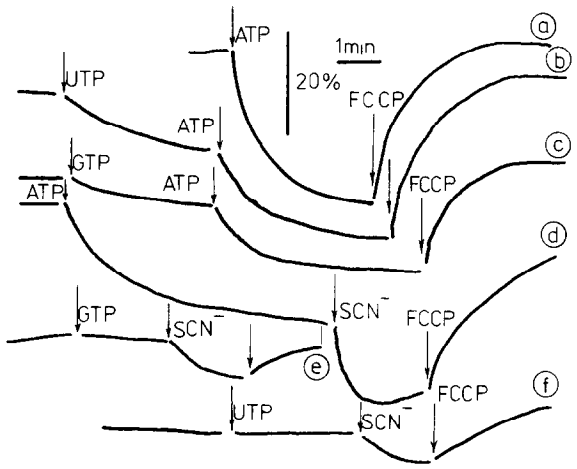

Fig.4. The effects of pH and SCN^- (10 mM) on a substrate specificity of the ΔpH generation across the membrane of isolated vacuoles: (a–c) pH 7.3; (d–f) pH 6.5.

nearly the original level indicating the collapse of ΔpH. NH_4Cl at 0.9–3.6 mM revealed the transient increase of fluorescence followed by its quenching. This points to the reversible ΔpH formation.

Vacuoles of *S. carlsbergensis* exhibited close ATP-, GTP- and UTPase activities, so we investigated the GTP- and UTP-dependent acidifications of the intravacuolar space too. Both GTP and UTP supported ΔpH formation at pH 7.3; however, ATP added after either of these nucleotides stimulated an additional ΔpH formation (fig.4b,c). UTP- and GTP-dependent ΔpH formation was not found at pH 6.5; however, the ATP-dependent proton pump did work (18% and 27% of fluorescence quenching at pH 6.5 and pH 7.3, respectively).

The experiments at pH 6.5 in the presence of SCN^- showed again that ATP was more effective than GTP and UTP in the intravacuolar acidification (fig.4d–f). Therefore, ATPase is the main proton pump of the vacuolar membrane and the participation of GTPase and UTPase in the ΔpH (and probably $\Delta\psi$) formation is insignificant.

4. DISCUSSION

This work shows that vacuoles of *S. carlsbergensis* possess the ATP-dependent proton pump. Its identification with the H^+-ATPase was impeded by the existence of the multitude of different

phosphohydrolases in the yeast vacuoles [5]. Therefore, it was unclear which enzyme did create ΔpH and $\Delta\psi$ across the tonoplast vesicles of *S. cerevisiae*: these vesicles exhibited nearly the same activities of ATPase, GTPase and UTPase (470, 440 and 270 nmol.mg protein^{-1}.min^{-1}, respectively [8]. In spite of the fact that these vesicles exhibited the ATP-dependent formation of ΔpH and $\Delta\psi$, GTP and UTP were not tested as the substrates of the H^+-pump [8]. Though GTPase and UTPase of *S. carlsbergensis* vacuoles have nearly the same or even higher activities as ATPase, ATP hydrolysis is more effective in the ΔpH (and probably $\Delta\psi$) generation (fig.4). This means that ATP hydrolysis is more strongly coupled to the $\Delta\mu H^+$ formation across the tonoplast than GTP or UTP.

The above results taken together with those on the ATPase purification [6,7] leave little doubt that vacuoles of *S. carlsbergensis* do possess electrogenic ATPase translocating protons inside these organelles.

Insensitivity of vacuolar ATPase to vanadate suggests that its fundamental mechanism of proton translocation may resemble that of F_1-like H^+-ATPases which are also insensitive to vanadate [13,14] and have never been shown to form a phosphorylated intermediate. Vacuolar ATPase of the yeast seems to resemble the proton pump of lysosomes [15] and chromaffin granules [16]. It differs from the enzyme of vacuo-lysosomes of Hevea in its sensitivity to DES, but seems to be similar to it in other properties [17].

The above data allowed us to suggest that K^+, Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+} and arginine are transported inside vacuoles by the cation/H^+ antiporters:

(i) Activation of ATPase by these cations (table 1);
(ii) Additional (or faster) generation of ΔpH by Zn^{2+} (fig.2a,c);
(iii) Additional formation of ΔpH by arginine which is prevented by KSCN (fig.3c–e);
(iv) More effective stimulation of ATPase by K_2SO_4 than KSCN (K_2SO_4, replacement of protons inside vacuoles by K^+ due to the postulated K^+/H^+ antiporter and activation of ATPase as a result; KSCN, $\Delta\psi$ conversion to ΔpH in the presence of permeant anion SCN^-).

Studies of the role of $\Delta\psi$ and ΔpH in the uptake of cations by yeast vacuoles as well as the physicochemical properties of vacuolar ATPase are underway.

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