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Chloride transport of yeast vacuolar membrane vesicles: a study of in vitro vacuolar acidification

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Effects of various solutes on acidification inside the vacuolar membrane vesicles of the yeast *Saccharomyces cerevisiae* were examined. ATP-dependent acidification was stimulated by the presence of chloride salts. There was essentially no difference in the stimulatory effects of NaCl, KCl, LiCl, and choline chloride. The membrane potential across the vacuolar membrane was reduced by the presence of Cl^- salts. Transport of $^{36}Cl^-$ is driven by the protonmotive force across the vacuolar membrane. Kinetic analyses have revealed that the stimulatory effect of Cl^- on internal acidification depends on two distinct components. One shows linear dependency on chloride concentration and is inhibited by 4,4′-diisothiocyano-2,2′-stilbenedisulphonic acid (DIDS). The other exhibits saturable kinetics with an apparent K_m for chloride of 15–20 mM. We conclude that the vacuolar membrane of yeast is equipped with Cl^- transport systems contributing to the formation of a chemical gradient of protons across the vacuolar membrane by shunting the membrane potential generated by proton translocation.

Introduction

Acidification of endomembrane systems including vacuoles, lysosomes, endosomes, coated vesicles and the Golgi apparatus has been demonstrated in various eukaryotic cells [1]. In the yeast *Saccharomyces cerevisiae* the vacuole is an acidic compartment that functions physiologically to store primary and secondary metabolites and to digest macromolecules [2–4]. We have found a number of transport systems in the vacuolar membrane: a H^+-translocating ATPase that generates a protonmotive force across the vacuolar membrane [5–7], and secondary transport systems for amino acids [8,9] and Ca^{2+} [10] driven by the protonmotive force. Their activities provide the vacuole with chemiosmotic functions for regulating ionic homeostasis in the cytosol [11–15]. Moreover, plant and fungal vacuoles contain various hydrolases for proteins, polysaccharides and polynucleotides [2,4], and these hydrolases are known to prefer low pH for maximal activity.

A class of V-type H^+-translocating ATPase [1,11, 14,15] participates as an electrogenic primary pump in transport of protons across the membrane, and thus generates an inside-positive membrane potential [5]. In theory, net proton translocation into the vacuole by the H^+-ATPase is limited by the protonmotive force loaded on a system, which is composed of both electric or chemical potential differences for protons. The proton pumping activity is balanced by some chemiosmotic force such that the inside-positive membrane potential at steady state prevents a net proton influx. This limits the formation of a chemical potential difference of protons across the membrane (ΔpH), i.e., acidification. Thus, questions arise as to how the vacuole is able to generate and maintain such an acidic environment under these limitations and what mechanism controls the acidification inside the vacuole. This paper describes evidence of the existence of two chloride transport systems which have essential roles in the ATP-dependent acidification inside the yeast vacuolar membrane vesicles.

Materials and Methods

Vacuolar membrane vesicles

Yeast vacuolar membrane vesicles were prepared from a haploid strain X2180-1A (Yeast Genetic Stock Center, Berkeley, CA) by the method of Ohsumi and Anraku [8]. The vacuolar membrane vesicles were sus-

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Abbreviations: ΔpH, chemical potential difference for protons; $\Delta\Psi$, membrane potential; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulphonic acid; Mes, 4-morpholineethanesulphonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

pended in 5 mM Mes-Tris (pH 7.2), 0.4 M sucrose and stored at −80°C before use. A modification of the method of Lowry et al. [16], which includes a precipitation step with 5% trichloroacetic acid, was used for protein assay, using bovine serum albumin as a standard.

Quenching assay of quinacrine and oxonol-V fluorescence

For measurement of quinacrine uptake [17], vacuolar membrane vesicles (about 30 μg of protein) were incubated in 2 ml of a solution (5 mM Mes-Tris (pH 7.2) and 5 mM $MgSO_4$) containing 5 μM quinacrine-2HCl and various test solutes at 25°C. Fluorescence of quinacrine was monitored by a spectrofluorometer (model MPF-4, Hitachi) at excitation and emission wavelengths of 425 and 495 nm, respectively. The reaction was initiated by addition of ATP-Tris (pH 7.2) to a final concentration of 0.5 mM. Quenching of oxonol-V fluorescence [18] was measured by incubating vacuolar membrane vesicles (about 50 μg of protein) in 2 ml of the same solution containing 0.5 μg/ml oxonol-V and various test solutes at 25°C. The reaction was initiated by addition of ATP-Tris (pH 7.2) to a final concentration of 0.5 mM, and fluorescence of oxonol-V was measured with the spectrofluorometer at excitation and emission wavelengths of 625 nm and 655 nm, respectively.

Assay of ATPase activity

The ATPase activity of the vacuolar membrane vesicles was determined by the method of Pazoles et al. [19] with modifications. Vacuolar membrane vesicles (about 20 μg ot protein) in 200 μl of 5 mM Mes-Tris (pH 7.2), 5 mM $MgSO_4$, 60 mM sorbitol and choline chloride, at concentrations indicated, were incubated with 0.5 mM [γ-^{32}P]ATP (0.7–3.7 MBq/mmol) for 120 s at 25°C in an Eppendorf tube, then 775 μl of ice-cold 5% (w/v) trichloroacetic acid containing 15% (w/v) activated charcoal (Norit A, Sigma) was added to the tube, mixed thoroughly and placed on ice for 0.5–1 h with occasional stirring. After centrifugation in a microfuge for 3 min, 500 μl of the supernatant was mixed with 2 ml of liquid scintillation cocktail ACS-II (Amersham, Arlington Heights, IL). This volume was assessed to be 60% of the total aqueous phase of the charcoal-treated reaction mixture measured by recovery of ^3H_2O (data not shown). The radioactivity of liberated [^{32}P]P_i was counted in a Beckman LS-9000 liquid scintillation counter.

Chloroquine uptake of the vacuolar membrane vesicles

Vacuolar membrane vesicles (about 20 μg of protein) were incubated in 1 ml of 5 mM Mes-Tris (pH 7.2), 5 mM $MgSO_4$, 60 mM sorbitol (or other solutes as indicated), 10 μM [^{3}H]chloroquine (3.7 MBq/mmol) and 0.5 mM ATP-Tris at 25°C. After incubation for 10 s or 120 s, the mixture was diluted into 3 ml of an ice-cold buffer containing 5 mM Mes-Tris (pH 7.2), 5 mM $MgSO_4$, 60 mM sorbitol or other solutes, immediately poured onto a nitrocellulose membrane filter (0.45μm pore size, code HATF, Milipore, Bedford, MA) and washed with the same buffer (1 ml, 3 times). Radioactivity retained on the filters was determined in 5 ml of liquid scintillation cocktail ACS-II in a Beckman LS-9000 liquid scintillation counter.

Chloride uptake of the vacuolar membrane vesicles

Vacuolar membrane vesicles (80 μg of protein) were incubated in tubes containing 100 μl of 5 mM Mes-Tris (pH 7.2), 5 mM $MgSO_4$, 60 mM sorbitol, 50 mM K^{36}Cl, and 0.5 mM ATP-Tris, 5 mM creatine phosphate, and 0.5 units of creatine kinase (Boehringer-Mannheim, Mannheim, Germany) at 25°C. At intervals of 1 min, the mixture was diluted into 2 ml of 5 mM Mes-Tris (pH 7.2), 5 mM $MgSO_4$, 60 mM sorbitol and 50 mM KCl, and poured onto a nitrocellulose membrane (code HATF, Milipore). The nitrocellulose membrane was washed with the same buffer (2 ml, 3 times) and radioactivity retained on the nitrocellulose membrane was determined as described above.

Reagents

[^{3}H]Chloroquine dihydrochloride (740 GBq/mmol) was purchased from New England Nuclear. [γ-^{32}P]-ATP (> 111 TBq/mmol) and [^{36}Cl]HCl (> 111 MBq/g Cl) were from Amersham. CCCP, and gramicidin S were obtained from Sigma. Oxonol-V was obtained from Nippon Kankoh Shikiso Kenkyusho (Okayama, Japan). All chemicals used were analytical grade.

Results

Anions stimulate the formation of a proton gradient (ΔpH), but reduce a membrane potential ($\Delta\Psi$) across the vacuolar membrane

Vacuolar membrane H^+-ATPase transports protons from the cytosol to inside the vacuole and generates a proton motive force across the vacuolar membrane [3,5]. The purified vacuolar membrane vesicles have right-side out orientation [8], thus the H^+-ATPase generates an influx of protons into the vesicles upon the addition of ATP. Weak bases such as chloroquine, quinacrine and methylamine are trapped in acidic compartments [17]. We examined the effects of various ions on formation of the chemical gradient of protons across the vacuolar membrane by using purified vacuolar membrane vesicles and radioactive chloroquine. Table I shows that the initial rate of chloroquine uptake was stimulated 1.5- to 1.7-fold by the presence of Cl^-, NO_3^- and SCN^-, but not of SO_4^{2-} or gluconate$^-$. No significant difference in stimulation was observed among the monovalent cations, Na^+, Li^+, and choline used as chloride salts. Although KCl showed a lower

TABLE I

Effects of solutes on chloroquine uptake by vacuolar membrane vesicles

The initial rate of chloroquine uptake of the vacuolar membrane vesicles was determined in 5 mM Mes-Tris (pH 7.2), 5 mM $MgSO_4$, 10 μM [^{3}H]chloroquine and the indicated solutes. Data represent mean ± S.E. for four determinations.

Solute added (mM)	Initial rate of chloroquine uptake (nmol/min per mg protein)	(%)
KCl (25)	55.7 ± 7.7	(150 ± 21)
Choline chloride (25)	66.5 ± 10.1	(179 ± 27)
NaCl (25)	65.9 ± 5.3	(177 ± 14)
LiCl (25)	62.4 ± 2.5	(168 ± 7)
KNO_3 (25)	60.6 ± 7.7	(163 ± 21)
KSCN (25)	56.1 ± 6.4	(151 ± 17)
K_2SO_4 (12.5)	40.0 ± 2.7	(108 ± 7)
K_2SO_4 (25)	40.2 ± 5.1	(108 ± 14)
Potassium gluconate (25)	36.3 ± 2.5	(98 ± 7)
Sorbitol (60)	37.2 ± 5.7	(100)

stimulatory effect than the other chloride salts, this slight difference was within experimental error.

ATP-dependent formation of a membrane potential ($\Delta\Psi$) across the vacuolar membrane in the presence of various solutes was examined by monitoring of quenching of oxonol-V fluorescence (Fig. 1). The fluorescence of oxonol-V was quenched immediately after the addition of ATP, indicating that an internal positive membrane potential was formed. In the presence of chloride (traces c and d), the membrane potential fell gradually and reached a steady level after hyperpolarization upon ATP addition. Quenching profiles of oxonol-V fluorescence were similar in the presence of

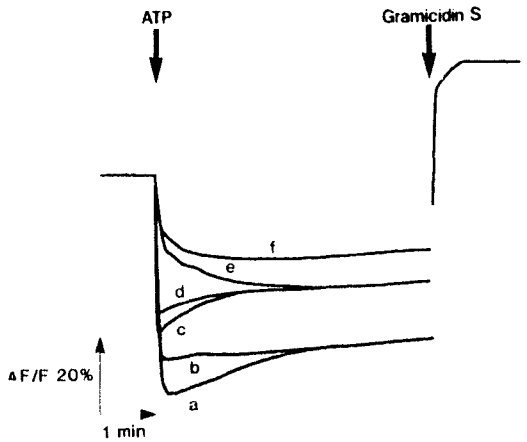

Fig. 1. Formation of $\Delta\Psi$ of vacuolar membrane in the presence of varied solutes. Membrane potential of vacuolar membrane vesicles was measured by quenching of oxonol-V quenching. The solution contained 5 mM Mes-Tris (pH 7.2), 5 mM $MgSO_4$, 60 mM sorbitol, 10 μg/ml oxonol-V, 50 μg of protein of vesicles and (a), none; (b), 50 mM potassium gluconate or 25 mM K_2SO_4; (c) 50 mM choline chloride; (d) 50 mM KCl or NaCl; (e) 50 mM KNO_3; (f) 50 mM KSCN. ATP-Tris (pH 7.2) (final concentration, 0.5 mM) and gramicidin S (10 μM) were added at the point indicated.

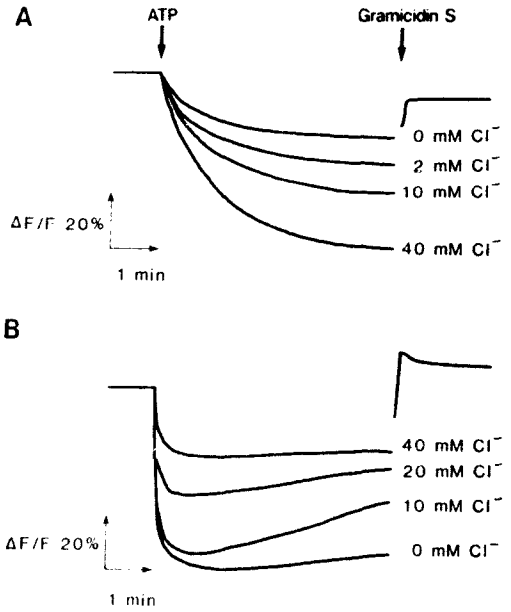

Fig. 2. Chloride-dependent formation of ΔpH and dissipation of $\Delta\Psi$. Formation of ΔpH was measured by quenching of quinacrine fluorescence in a solution containing 28 μg protein of membrane vesicles, 5 mM Mes-Tris, pH 7.2, 5 mM $MgSO_4$, 60 mM sorbitol, 5 μM quinacrine-2HCl and various concentrations of choline chloride. Formation of $\Delta\Psi$ at various concentrations of choline chloride was measured by quenching of fluorescence of oxonol-V.

KCl, NaCl (trace d) and LiCl (data not shown), but were slightly different when choline chloride was added. On the other hand, the profiles with NO_3^- and SCN^- were apparently different: the initial hyperpolarization was not observed, and the membrane was less polarized than those in the presence of chloride salts. When the reaction mixture contained sorbitol alone or sorbitol and gluconate, the steady level of the membrane potential was larger than those in the presence of chloride, nitrate, or thiocyanate salts.

These observations (Table I and Fig. 1) show that the ΔpH increased in the presence of chloride, nitrate and thiocyanate salts, whereas the $\Delta\Psi$ reduced. We predicted that this phenomenon indicated that anions such as Cl^-, NO_3^- and SCN^- permeate into the vacuolar membrane vesicles and dissipate the internal positive membrane potential and facilitate the acidification inside the vesicles.

The acidification is enhanced by two components of Cl^- transport

We then examined a mechanism(s) of the formation of the ΔpH across the vacuolar membrane. Acidification inside the vacuolar membrane vesicles results in accumulation of a weak base, quinacrine, inside the vesicles, which results in quenching of the fluorescence of quinacrine [5,14,15]. Fig. 2 (upper panel) shows that rapid quenching of quinacrine fluorescence took place

immediately after addition of ATP and reached a steady level in about 5 min. An ionophore, gramicidin S, reversed the fluorescence intensity mostly to its initial level. Both the initial and maximum levels of quenching ($\Delta F/F$) correlated linearly with the amount of the vesicle protein within the range of 20–60 μg under standard assay conditions (data not shown). The extents of vesicular acidification (Fig. 2, upper part) and membrane potential formed (Fig. 2, lower part) were measured in the presence of varied concentrations of choline chloride. The initial rates and steady levels of quinacrine quenching, which reflect transport of protons and formation of ΔpH, were stimulated, whereas the membrane potential was reduced with increase of the salt concentration.

Stimulation on the initial rate of quinacrine quenching by Cl^- did not saturate by increasing the salt concentration upto 100 mM (Fig. 3, closed circles). It seemed to be composed of two kinetically different parameters: one component was saturated at approx. 30 mM choline chloride, whereas the other maintained a linear relationship with choline chloride concentration. Therefore, the chloride stimulation was formulated as:

$$V_{obs} = V_{max}[Cl^-]/(K_m + [Cl^-]) + k[Cl^-] + V_0$$

where: V_{obs}, the initial rate of quenching; V_{max}, the maximum velocity of quenching (owing to a saturable component); V_0, the initial rate of quenching at 0 mM choline chloride (due to a component independent of Cl^-); K_m, an apparent Michaelis constant for Cl^- (owing to a saturable component); k, the rate constant for Cl^- of the linear component.

Least square fitting, using a microcomputer, estimated V_{max}, V_0, K_m and k as shown in Table II. The saturable component showed an apparent K_m for choline chloride of 20 mM. The ratio of V_0 to V_{max} was 1.0, indicating that the saturable component is responsible for 2-fold stimulation at a saturated concentration of choline chloride. Kinetic analysis of radioactive

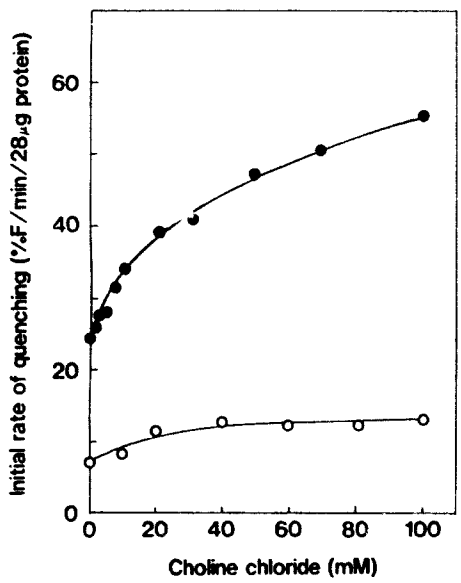

Fig. 3. Kinetics of Cl stimulation on the ΔpH formation. The initial rates of quenching of quinacrine fluorescence in the presence of varied concentrations of choline chloride were plotted. The closed circles and open circles indicate the activity of untreated and DIDS-treated vacuolar membrane vesicles, respectively.

chloroquine uptake into the vacuolar membrane vesicles also showed the existence of two distinctive components with a similar dependency on choline chloride (Table II). In this case, the apparent K_m for Cl^- was approx. 15 mM, and the ratio of V_0/V_{max} was approx. 0.8.

Effect of Cl^- on the vacuolar H^+-ATPase activity

The protonmotive force across the vacuolar membrane is to be limited by the activity of the H^+-ATPase. Thus, the stimulation of ΔpH formation by Cl^- may be due to a change in the H^+-ATPase activity. We determined the H^+-ATPase activities in the presence of varied concentrations of choline chloride by measuring the ATP hydrolysis activities of the vacuolar membrane

TABLE II

Kinetic parameters of chloride stimulation on vacuolar ATPase and acidification activities

Activities of vacuolar ATPase and acidification were determined under various conditions. Kinetic parameters determined were summarized.

		Acidification		ATPase
		quinacrine fluorescence	chloroquine uptake	ATP hydrolysis
Saturable component	V_{max}	23.5 [a]	47.3 [b]	0.077 [c]
	K_m	19.3 mM	15.3 mM	8.6 mM
Linear component	k	0.12 [d]	0.16 [c]	not detected
Cl^- independent	V_0	24.0 [a]	36.6 [b]	0.29 [c]

[a] $(\%\Delta F/F)$/min per 28 μg protein; [b] nmol uptake/min per mg protein; [c] nmol P_i/min per mg protein; [d] $(\%\Delta F/F)$/min per 28 μg protein/mM Cl^-; [e] nmol uptake/min per mg protein/mM Cl^-.

vesicles. As shown in our previous studies, the ATP hydrolysis is known to be carried out by the H^+-ATPase because the vacuolar membrane vesicles from H^+-ATPase-deficient mutants do not demonstrate ATP hydrolysis activity [14,15].

Fig. 4 shows that the ATP hydrolysis activity increased slightly as the concentration of choline chloride increased. This stimulation of the ATPase activity by Cl^- was composed of only one saturable component, which exhibited an apparent K_m for Cl^- of 8 mM, and no linear component was found (Table II). The V_0/V_{max} value was determined to be approx. 4 (Table II), indicating that the H^+-ATPase is not affected substantially by increasing the concentration of Cl^-, as in Fig. 4. Therefore, neither the linear nor saturable component of Cl^- stimulation of the vacuolar acidification, which has a V_0/V_{max} value of 0.8–1, was attributable to the Cl^- stimulation of the H^+-ATPase. Thus, we concluded that this stimulation of the H^+-ATPase activity by Cl^- was not related to any mechanism of the Cl^--stimulated formation of the ΔpH.

Transport of radioactive chloride into the vacuolar membrane vesicles

The results shown above strongly suggested that Cl^- transport was required for the ATP-dependent acidification of vacuolar membrane vesicles. To demonstrate this possibility directly, we examined $^{36}Cl^-$ uptake by the vacuolar membrane vesicles. Fig. 5 shows the time-course of Cl^- transport into the vesicles and its inhibition by a protonophore, CCCP, indicating that the protonmotive force generated across the vacuolar membrane drove active transport of chloride.

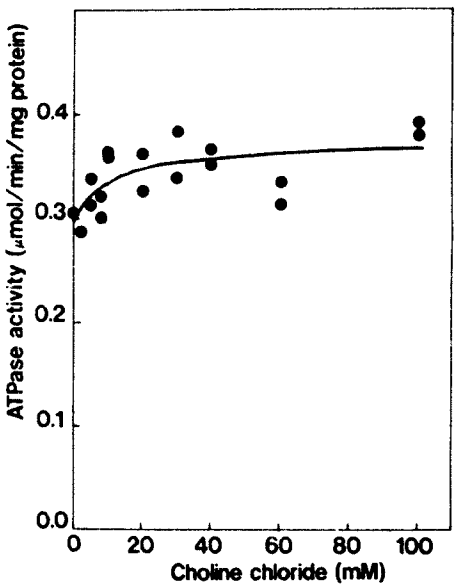

Fig. 4. Effect of Cl^- on vacuolar H^+-ATPase activity. The ATP hydrolysis activity of vacuolar membrane vesicles was determined in the presence of various concentrations of choline chloride.

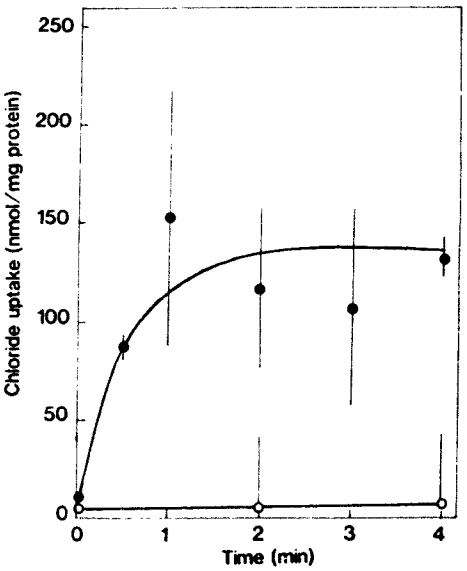

Fig. 5. Chloride uptake by the vacuolar membrane vesicles. The vacuolar membrane vesicles were incubated with 50 mM $K^{36}Cl$ and ATP-regenerating system, in the presence of 10 μM CCCP (○) or in the absence of CCCP (●). Bars indicate standard deviations ($n = 4$, except that $n = 2$ at 30 s).

DIDS inhibits the linear component of Cl^- transport

DIDS is a reagent known to change the anion permeability of plant vacuolar membranes [20–22]. Yeast vacuolar membrane vesicles were treated with varied concentrations of DIDS for 3 h on ice, then their activities of ATP hydrolysis and acidification were determined (Fig. 6): treatment with 10–50 μM DIDS reduced both activities significantly. We then compared the acidification activity of the DIDS-treated vesicles in the presence of 25 mM choline chloride and found that the Cl^- stimulation on acidification was also inhibited by treatment with DIDS. Stimulation of about 1.6-fold was observed with DIDS-untreated vesicles, whereas it was less than 1.3-fold with the DIDS-treated vesicles (Fig. 6). This result indicates that DIDS partially inhibited the Cl^- stimulation of ΔpH formation.

The kinetic effect of Cl^- on acidification of the DIDS-treated vesicles (20 μM) is also shown in Fig. 3 (open circles). Computer analysis of the data revealed that this stimulation was composed of only one saturable component. An apparent K_m for Cl^- was determined to be 19.3 mM, and V_0/V_{max} was 0.89. These values were consistent with the kinetic parameters of the saturable component of Cl^- stimulation observed with vesicles not treated by DIDS. We suggest there-

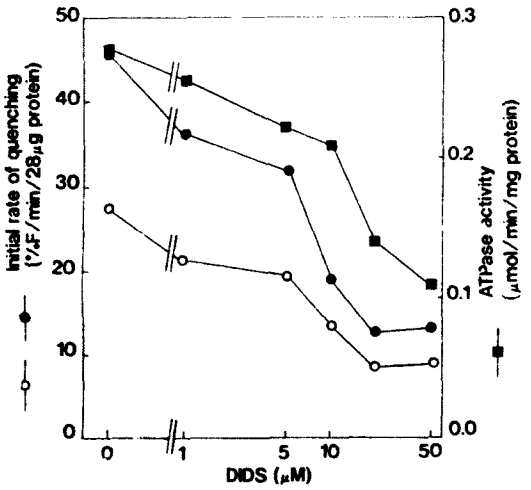

Fig. 6. Effect of DIDS on H^+-ATPase and acidification activities of the vacuolar membrane vesicles. The vacuolar membrane vesicles were treated with various concentrations of DIDS in 5 mM Mes-Tris (pH 7.2), 0.4 M sucrose for 3 h on ice. The initial rates of quenching of quinacrine uptake were determined in the presence of 25 mM choline chloride (○) and in the absence of choline chloride (●). The H^+-ATPase activity of the DIDS-treated vesicles is shown by closed squares.

fore that DIDS affected the linear but not the saturable component of Cl^- transport. The fact that the linear component of Cl^- transport is inhibited by DIDS strongly suggests that it is not a simple diffusion process due simply to leakiness of the lipid bilayer membrane for chloride.

Discussion

An interior acidic environment in the yeast vacuole is important for such vacuolar functions as storage and digestion. We examined acidification inside the purified vacuolar membrane vesicles of yeast, which have previously been shown to be essentially free from contamination of mitochondria and other organelles [5,8]. Four main results are described in this paper. (1) Acidification inside the vesicles was stimulated, whereas membrane potential was decreased, in the presence of increased concentrations of chloride. (2) The vacuolar membrane H^+-ATPase activity was neither stimulated nor inhibited significantly by the presence of Cl^-. (These two results, taken together, indicate that transport of chloride across the membrane is a prerequisite for acidification inside the vesicles.) (3) In fact, the vacuolar membrane vesicles took up $^{36}Cl^-$ and this transport was driven by the protonmotive force. (4) Two components for Cl^- transport were detected kinetically, which showed different sensitivities for the inhibitor DIDS.

Our previous studies [5,14,15] have shown that vacuolar acidification in yeast cells is regulated by proton influx, driven by the vacuolar membrane H^+-ATPase. This raises the question of how this proton transport establishes acidification inside the vacuole. Vacuoles are a storage compartment for various solutes, including basic amino acids and polyphosphates [2,12,13]. These solutes behave as buffers for proton, thus a large proportion of translocated protons are buffered with these solutes and less would remain as active free protons in the lumen of vacuoles. While electric charges of protons are translocated into the vacuolar lumen, a membrane potential is generated. This electro-osmotic event, in principle, must result in the formation of a large membrane potential, with only a small pH gradient across the vacuolar membrane.

We investigated the mechanisms involved in dissipating the membrane potential generated by H^+ translocation across the vacuolar membrane. We conclude that anion transport across the vacuolar membrane plays an essential role in establishing and maintaining the vacuolar acidification. First, the formation of ΔpH was stimulated whereas the interior-positive membrane potential is reduced by the presence of chloride. Second, transport of chloride into the vesicles takes place during the acidification inside the vesicles. We have focused on the effects of chloride, even though nitrate and thiocyanate stimulated acidification as well, because thiocyanate is an unphysiological anion and nitrate is a potent inhibitor for V-type ATPases [3,6]. Kane et al. [24] reported that subunits of the yeast vacuolar membrane H^+-ATPase dissociate from the membrane in the presence of high concentrations of chaotropic anions, including nitrate. Phosphate ions may also participate in vacuolar acidification, as this anion is known to be dynamically compartmentalized between the cytosol and the vacuole [2,4,23]. However, we did not examine the effect of phosphate in this study because the substantial buffering capacity of phosphate anions around neutral pH makes it difficult to assess acidification inside the vesicles with pH probes, such as quinacrine and chloroquine.

Vacuolar acidification profiles did not show any obvious difference in the presence of different salts, including KCl, NaCl, LiCl and choline chloride. This indicates that Cl^- plays a major role in the stimulation of the acidification. However, specific cations slightly affect the rate of dissipation of the membrane potential. In the presence of KCl or NaCl, the hyperpolarized state was not apparent, whereas in the presence of choline chloride, a large membrane potential was formed immediately after the addition of ATP, followed by slow dissipation to the steady-state level (see Fig. 2, lower part). We have previously described the properties of a membrane potential-dependent cation channel in the yeast vacuolar membrane [25]. This

channel has a broad selectivity for monovalent cations including K^+, Na^+, Li^+, but not for choline. The different effect of choline chloride on the membrane potential suggests that a monovalent cation flux through the channel may partly contribute to the process of dissipating the membrane potential.

Our kinetic analyses (Fig. 3) have revealed the existence of two components for Cl^- stimulation on acidification. Furthermore, these two components show different sensitivities for DIDS. We hypothesize from these observations that two separate Cl^- transport systems are involved in acidification: the saturable component may represent the activity of a carrier-type transport system that has an apparent K_m for Cl^- around 20 mM, and the linear one may be due to the activity of a channel-type system that is sensitive for DIDS. This model should be examined further for a understanding of the molecular mechanisms of vacuolar acidification.

Rothman et al. [26] have reported that some mutations affecting the process of vacuolar protein sorting (*vps* mutations) result in defects in vacuolar acidification. We suggest that the mutations not only on structural or regulatory genes for the H^+-ATPase but also on those for Cl^- transport systems may cause defects in vacuolar acidification, since substantial acidification inside the vacuole requires both H^+ and Cl^- transport, as shown here. Recently, Preston et al. [27] have developed an in vivo assay for pH in yeast vacuoles using a fluorescent pH-sensitive dye and microscope. They have also reported that vacuoles in the *pep12* mutant maintain nearly normal pH, but fail to accumulate quinacrine. They suggested that this phenotype results from a decreased capacity for proton accumulation inside the vacuoles. We propose that there are two transport systems for Cl^- in the vacuole. Thus, mutation in one of the Cl^- transport systems may show a partial acidification defect, like that seen in the *pep12* mutation.

Eukaryotic cells have acquired at least two physiological mechanisms in order to control organelle-cytosol interaction for establishing acidic organelle compartments. In mammalian endosomes, cations move from the lumen out into the cytosol during ATP-dependent acidification [19,28]. In plant vacuoles [20–22,29] and the Golgi apparatus in mammalian cells [30], anion flux takes place during ATP-dependent acidification. Our investigation shows that yeast vacuoles, like plant vacuoles, use anion transport for conversion of the $\Delta\Psi$ into the ΔpH.

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