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MINI-REVIEW

Structure and Function of the Yeast Vacuolar Membrane Proton ATPase

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Abstract

Our current work on a vacuolar membrane proton ATPase in the yeast *Saccharomyces cerevisiae* has revealed that it is a third type of H^+-translocating ATPase in the organism. A three-subunit ATPase, which has been purified to near homogeneity from vacuolar membrane vesicles, shares with the native, membrane-bound enzyme common enzymological properties of substrate specificities and inhibitor sensitivities and are clearly distinct from two established types of proton ATPase, the mitochondrial F_0F_1-type ATP synthase and the plasma membrane E_1E_2-type H^+-ATPase. The vacuolar membrane H^+-ATPase is composed of three major subunits, subunit *a* (M_r = 67 kDa), *b* (57 kDa), and *c* (20 kDa). Subunit *a* is the catalytic site and subunit *c* functions as a channel for proton translocation in the enzyme complex. The function of subunit *b* has not yet been identified. The functional molecular masses of the H^+-ATPase under two kinetic conditions have been determined to be 0.9–1.1 $\times$ 10^5 daltons for single-cycle hydrolysis of ATP and 4.1–5.3 $\times$ 10^5 daltons for multicycle hydrolysis of ATP, respectively. *N*, *N'*-Dicyclohexylcarbodiimide[2] does not inhibit the former reaction but strongly inhibits the latter reaction. The kinetics of single-cycle hydrolysis of ATP indicates the formation of an enzyme–ATP complex and subsequent hydrolysis of the bound ATP to ADP and Pi at a 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole-sensitive catalytic site. Cloning of structural genes for the three subunits of the H^+-ATPase (*VMA1*, *VMA2*, and *VMA3*) and their nucleotide sequence determination have been accomplished, which provide greater advantages for molecular biological studies on the structure–function relationship and biogenesis of the

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[2]Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; DCCD, *N*,*N'*-dicyclohexylcarbodiimide; DES, diethylstilbestrol; DIDS, 4,4'-diisothiocyano-2,2'-stilbene disulfonic acid; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; Pi, inorganic phosphate; SDS, sodium dodecylsulfate; SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; SITS, 4-acetamide-4'-isothiocyanatostilbene-2,2'-disulfonic acid; ZW3-14, *N*-tetradecyl-*N*,*N'*-dimethyl-3-ammonio-1-propanesulfonate.

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enzyme complex. Bioenergetic aspects of the vacuole as a main, acidic compartment ensuring ionic homeostasis in the cytosol have been described.

Key Words: Vacuolar membrane H^+-ATPase; vacuoles; *Saccharomyces cerevisiae*; catalytic cooperativity of ATP hydrolysis; *VMA* genes.

Introduction

Vacuoles are persistent organelles in the cells of higher plants, algae, and fungi. About a century ago De Vries (1885) described the morphological appearance of these organelles and gave the name "vacuole," which did not stain with eosin and so gave an image of "vacuo" cell sap, encircled by a extensible structure, the tonoplast (from *tonos*). The tonoplast, or vacuolar membrane, is composed of a single bilayer made of phospholipids and cholesterol, and the volume of vacuoles amounts to 20–30% of the total volume of yeast cells and 80–90% that of algae.

The characteristics of yeast vacuoles as a digestive or vacuo-lysosomal compartment were demonstrated in the work of Matile (1975). The vacuolar sap is acidic at pH 5.5 and contains a number of proteases, nucleases, glycosidases, and phosphatases in a cryptic state. The vacuole also establishes a differential storage compartment for basic amino acids and Ca^{2+} ions, which are sequestered from the cytosol by specific nH^+/solute antiport systems (Anraku, 1987a,b). During the last ten years, it has become known that a new, distinct class of proton-translocating ATPase exists ubiquitously in vacuo-lysosomal and endomembranous organelles including fungal and plant vacuoles, animal lysosomes, endosomes, Golgi bodies, and chromaffin granules (Al-Awqati, 1986; Anraku, 1987a; Bowman and Bowman, 1986; Manolson *et al.*, 1987; Nelson, 1988; Ohkuma, 1987; Rudnick, 1986; Sze, 1985). The primary role of a family of this type of H^+-ATPases has been ascribed to ATP-dependent membrane energization and acidic compartmentation, which enable the organelles to drive active transport of various solutes or to control digestive and processing functions of hydrolytic enzymes (Anraku, 1987b; Mellman *et al.*, 1986).

This article addresses biochemical and bioenergetic views of yeast vacuolar membrane H^+-ATPase, whose function is to confer to the vacuole an autonomic energization system for establishing an acidic compartment with the ability to regulate ionic homeostasis in the cytosol.

Discovery to Vacuolar Membrane H^+-ATPase

Biochemical studies of the vacuolar membrane in yeast cells originated with the work of Ohsumi and Anraku (1981), who established a simple

method for separating intact vacuoles of high purity from *Saccharomyces cerevisiae*, This procedure included spheroplasting of cells followed by differential separation of vacuoles in the crude lysate by flotational centrifugation in discontinuous Ficoll-400 gradients and allows 28-fold enrichment of α-mannosidase, a marker of the vacuolar membrane (Yoshihisa *et al.*, 1988). A purified preparation contains less than 0.05% each of the marker enzyme activities of glucose-6-phosphate dehydrogenase for cytosol, succinate dehydrogenase for mitochondria, NADH-cytochrome *c* reductase for microsomes, and chitin synthetase for plasma membrane (Kakinuma *et al.*, 1981). In addition, it contains considerable amounts of markers for vacuolar sap, such as arginine, polyphosphates, and alkaline phosphatase, indicating that the organelles remain intact during the isolation procedure. A brief method for preparing vacuolar membrane vesicles with a right-side-out orientation has been developed using the purified intact vacuoles (Ohsumi and Anraku, 1981). This procedure removes most of the arginine, polyphosphates, and alkaline phosphatases from the intact vacuoles, but scarcely affects their α-mannosidase activity (Kakinuma *et al.*, 1981).

Kakinuma *et al.*, (1981) found that the preparation of vacuolar membrane vesicles shows an unmasked Mg^{2+}-ATPase activity with an optimal pH of 7.0. The enzyme requires Mg^{2+} ion but not Ca^{2+} ion for its activity and hydrolyzes ATP, GTP, UTP, and CTP in this order. The K_m value for ATP was determined to be 0.2 mM. ADP and AMP are not hydrolyzed by the enzyme (Kakinuma *et al.*, 1981; Uchida *et al.*, 1985). The activity is sensitive to DCCD, which is the potent inhibitor of a family of H^+-translocating ATPases. The activities of intact vacuoles and of vacuolar membrane vesicles are stimulated 3- and 1.5-fold, respectively, by the protonophore uncoupler SF6847 and the K^+/H^+ antiporter ionophore nigericin.

ATP hydrolysis-dependent uptake of protons into vacuolar membrane vesicles has been demonstrated directly by the change in quenchings of 9-aminoacridine and quinacrine fluorescences. The electrochemical potential difference of protons across the vacuolar membrane generated upon ATP hydrolysis was determined by a flow-dialysis method with [^{14}C]methylamine for measuring the formation of ΔpH and with [^{14}C]KSCN for measuring the membrane potential. The protonmotive force (ΔP) thus calculated is 180 mV, with contribution of 1.7 pH units, interior acid, and of a membrane potential of 75 mV, interior positive (Kakinuma *et al.*, 1981). It is concluded that the Mg^{2+}-ATPase of vacuolar membranes is a new DCCD-sensitive, H^+-translocating ATPase.

We have raised monoclonal antibodies against subunits *a* and *b* of the H^+-ATPase (unpublished results). Indirect immunofluorescence staining of yeast cells with R70, a monoclonal antibody against the subunit *a*, shows that the enzyme complex is strictly located on the cytoplasmic side of vacuoles (Fig. 1).

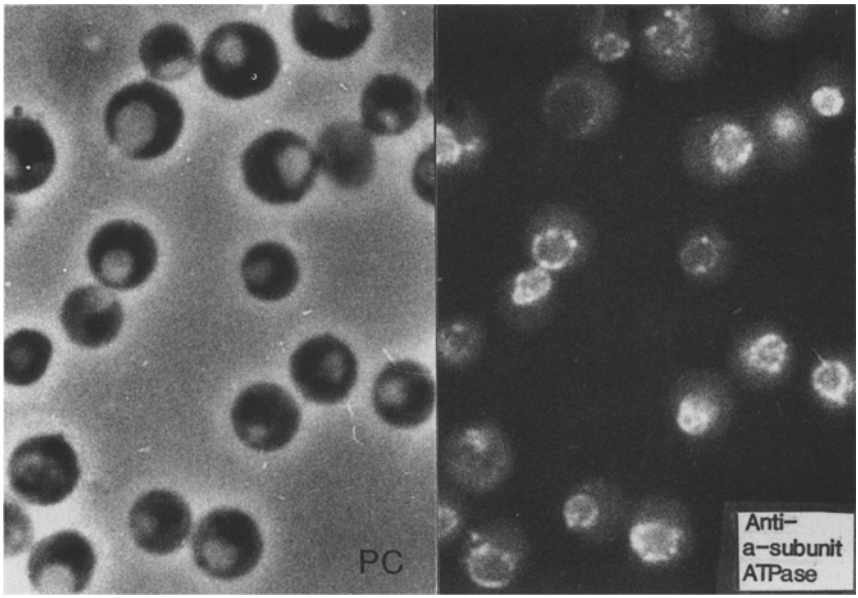

Fig. 1. Immunofluoresence detection of the H^+-ATPase on vacuoles in yeast cells. Cell of *S. cerevisiae* haploid strain X2180-1A were grown in YPD medium, harvested at the middle exponential phase of growth, converted into spheroplasts, and subjected to indirect immunofluorescence staining with the monoclonal antibody R70 raised against the subunit *a* of the enzyme. Left pannel: cell morphology examined by phase-contrast photomicrography. Central vacuoles are seen as spherical bodies with brighter contrast in the cytoplasm; Right pannel: fluorescence microscopic view of the same cells, showing distinct localization of the enzyme on the membrane of central vacuoles.

Enzymatic Properties of the Purified Vacuolar Membrane H^+-ATPase

The vacuolar membrane H^+-ATPase can be solubilized efficiently by the zwitterionic detergent ZW3-14 and purified to near homogeneity by glycerol density gradient centrifugation (Uchida *et al.*, 1985, 1988b). Detergents such as cholate, Sarkosyl, Triton X-100, and Tween 80 are not effective. Treatment of isolated vacuolar membrane vesicles with 1 mM EDTA before solubilization of the enzyme is effective, because this EDTA-wash removes most of the acid and alkaline phosphatases, which are soluble marker enzymes of the vacuolar sap and associated loosely with the vacuolar membrane, without affecting the ATPase activity (Uchida *et al.*, 1988b).

The vacuolar membrane "three-subunit" H^+-ATPase thus purified requires phospholipids for maximal activity and shows a specific activity of 16–18 units/mg protein in the presence of 0.1 mg/ml of asolectin (Uchida *et al.*, 1985). Table I summarizes the properties of purified vacuolar membrane

Table I. Enzymatic Properties of Three H^+-ATPases of *S. cerevisiae*

		Relative activity (%)[a]			
		Purified vacuolar membrane H^+-ATPase	Vacuolar membrane H^+-ATPase	Mitochondrial H^+-ATPase	Plasma membrane H^+-ATPase
A. Optimal pH		6.9	7.0	8.5	5.5
B. Substrate	(mM)				
ATP	(1)	100	100	100	100
GTP	(1)	68	56	147	29
UTP	(1)	22	26	33	15
CTP	(1)	19	22	1.6	12
C. Inhibitor	(mM)				
None		100	100	100	100
Sodium azide	(2.0)	95	110	4	105
Sodium vanadate	(0.1)	95	96	100	16
KNO_3	(50)	57	55	96	100
DCCD	(0.001)	38	63	12	86
NBD-Cl	(0.1)	23	27	6	79
Tributyltin	(0.1)	14	45	15	33
SITS	(0.004)	36	44	69	23
Oligomycin	(0.047)	96	74	10	74
Miconazole	(0.2)	106	109	46	23
DES	(0.1)	30	48	95	16
Quercetin	(0.1)	37	67	100	30
$CaCl_2$	(0.1)	101	98	91	82
$NiCl_2$	(0.5)	78	77	93	96
$CuCl_2$	(0.5)	10	12	99	1
$HgCl_2$	(0.5)	10	3	13	1
$ZnCl_2$	(0.5)	45	36	65	47
$LaCl_3$	(0.5)	66	95	71	39

[a]Assays were carried out with 1 mM substrate, 1 mM $MgCl_2$, 25 mM MES/Tris, and the inhibitor indicated at pH 6.9 (vacuolar membrane H^+-ATPase) pH 8.9 (mitochondrial H^+-ATPase), or pH 6.0 (plasma membrane H^+-ATPase).

H^+-ATPase and H^+-ATPases from vacuolar membranes, plasma membranes, and mitochondria from *S. cerevisiae* (Anraku *et al.*, 1987; Uchida *et al.*, 1985, 1988b). The purified vacuolar membrane H^+-ATPase in the presence of phospholipids has the same pH optimum (pH 6.9) and K_m value for ATP hydrolysis (0.21 mM) as the native, membrane-bound enzyme. ADP is not hydrolyzed and inhibits the enzyme activity noncompetitively, with a K_i value of 0.31 mM. AMP and *p*-nitrophenyl phosphate are neither substrates nor inhibitors. The activity of the purified enzyme is not inhibited by antiserum against yeast mitochondrial F_1-ATPase and yeast mitochondrial F_1-ATPase inhibitor protein (Uchida *et al.*, 1985).

The vacuolar membrane H^+-ATPase is a nitrate-sensitive enzyme and not inhibited by azide and vanadate (Uchida *et al.*, 1988b). The activity is

moderately inhibited by 50 mM KNO_3, whereas it is not affected at all by 2 mM NaN_3 or 0.1 mM Na-vanadate (Table I): Note that NaN_3 and Na-vanadate are the potent inhibitors of mitochondrial F_0F_1-H^+-ATPase and plasma membrane H^+-ATPase, respectively (Anraku *et al.*, 1987; Uchida *et al.*, 1988b). In this respect, these three anions are very effective chemicals for enzymological discrimination of three types of H^+-ATPase in yeast cells.

Like the F_0F_1-ATPase and plasma membrane ATPases, the vacuolar membrane H^+-ATPase is a proton-translocating ATPase (Kakinuma *et al.*, 1981) and is inhibited strongly by DCCD (Table I). The K_i values for DCCD of the purified vacuolar enzyme and ATPases in vacuolar membranes, submitochondrial particles, and plasma membranes from *S. cerevisiae* have been determined to be 0.8, 2, 0.2, and 8 μM, respectively (Uchida *et al.*, 1985, 1988b). It has been found that the inhibitor sensitivities of the vacuolar membrane H^+-ATPase are unique (see Table I): The activity is strongly inhibited by NBD-Cl, tributyltin, and SITS, which are inhibitors of F_0F_1-type ATPases from mitochondria and/or chloroplasts, but no by oligomycin. It is not affected at all by miconazole, which is an inhibitor of E_1E_2-type ATPases from plasma membranes, but is moderately inhibited by DES and quercetin, which are inhibitors of Na^+/K^+-ATPases.

There are several recent reports of the partial purification of H^+-ATPases from plant and fungal vacuolar membranes (Bowman *et al.*, 1986; Kaestner *et al.*, 1988; Lichke and Okorokov, 1985; Mandala and Taiz, 1985; Manolson *et al.*, 1985; Marin *et al.*, 1985; Randall and Sze, 1987; Rea *et al.*, 1988). These H^+-ATPases resemble one another enzymatically and appear to be composed of three major polypeptides, like the enzyme from *S. cerevisiae* (Bowman *et al.*, 1986; Lai *et al.*, 1988; Mandala and Taiz, 1985).

Molecular Organization of the Vacuolar Membrane H^+-ATPase

The purified vacuolar membrane H^+-ATPase is composed of three major subunits, subunit *a* (M_r = 67 kDa), *b* (M_r = 57 kDa), and *c* (M_r = 20 kDa), which are determined electrophoretically on 10% polyacrylamide gel in the presence of SDS (Hirata *et al.*, 1989): These values were formerly reported to be 89, 64, and 20 kDa, respectively, which all were determined on 15% polyacrylamide gel in the presence of SDS (Uchida *et al.*, 1985).

Subunit *a* is predicted to be the catalytic site of the enzyme complex by following affinity-labeling and chemical modification experiments (Uchida *et al.*, 1988a): (1) The ATP analogue 8-azido-ATP binds to subunit *a* specifically in an ATP-inhibitable manner. (2) NBD-Cl, which is known to interact specifically with the β-subunit (a catalytic polypeptide) of mitochondrial F_0F_1-ATPase, can bind covalently to subunit *a* in an ATP-protectable

manner. (3) This chemical reaction, which modifies a single tyrosine residue in the subunit molecule, results in enzyme inactivation and loss of ability to form an enzyme–ATP complex, the first intermediate in the hydrolytic reaction.

Subunit *c* has been identified as a DCCD-binding protein (Uchida *et al.*, 1985) and functions as a channel for proton translocation in the enzyme complex (Kakinuma *et al.*, 1981). Subunit *b* seems to be present in an equimolar amount with subunit *a* in the purified "three-subunit" H^+-ATPase, but its function has not yet been identified.

Recently, the functional molecular mass of the yeast vacuolar membrane H^+-ATPase under steady-state conditions for ATP hydrolysis has been determined to be $4.1–5.3 \times 10^5$ daltons (Hirata *et al.*, 1989). This observation indicates that the enzyme is oligomeric in terms of a catalytic site. There have also been reports that the vacuolar membrane H^+-ATPases in plants and *Neurospora* have similar large molecular sizes of 400–520 kDa (Bowman *et al.*, 1986; Lai *et al.*, 1988; Mandala and Taiz, 1985). Then, if it is assumed, based on these observations and other available data, that the vacuolar type ATPases are mainly composed of three subunits, subunit *a* (67–72 kDa), *b* (57–62 kDa), and *c* (16–20 kDa) (Bowman *et al.*, 1985; Hirata *et al.*, 1989; Lai *et al.*, 1988; Mandala and Taiz, 1985), the oligomeric ATPase complex may have the molecular composition $(a, b)_m c_n$, where $m \geqslant 3$ and $n > m$. Possibilities cannot be excluded, however, that the H^+-ATPase complex is associated with some other unidentified subunits, whose function(s) are not necessarily essential for *in vitro* ATPase activity as "the three-subunit" enzyme is identical to the native, membrane-bound enzyme enzymologically and kinetically (Uchida *et al.*, 1985, 1988b).

Reaction Mechanism of ATP Hydrolysis

The reaction mechanism of the vacuolar membrane H^+-ATPase has been studied under two kinetic conditions (Uchida *et al.*, 1988a, Hirata *et al.*, 1989). For assay of the steady-state (multi-cycle) hydrolysis of ATP, purified enzyme (4 μg of protein or 23 nM of holo H^+-ATPase) was incubated with 5 mM ATP at 30°C, whereas for assay of the non-steady-state (single-cycle) hydrolysis of ATP, 230 nM of purified H^+-ATPase was incubated with 40 nM [γ-^{32}P]ATP (Uchida *et al.*, 1988a). Under this non-steady-state condition, the amounts of total Pi formed and [γ-^{32}P]ATP bound to a single catalytic site and committed to hydrolysis can be determined by the method of Grubmeyer *et al.*, (1982). From these experiments, the following elementary steps of ATP hydrolysis are revealed (see Fig. 2): (1) ATP initially binds to the NBD-Cl sensitive catalytic site to form an enzyme–ATP complex.

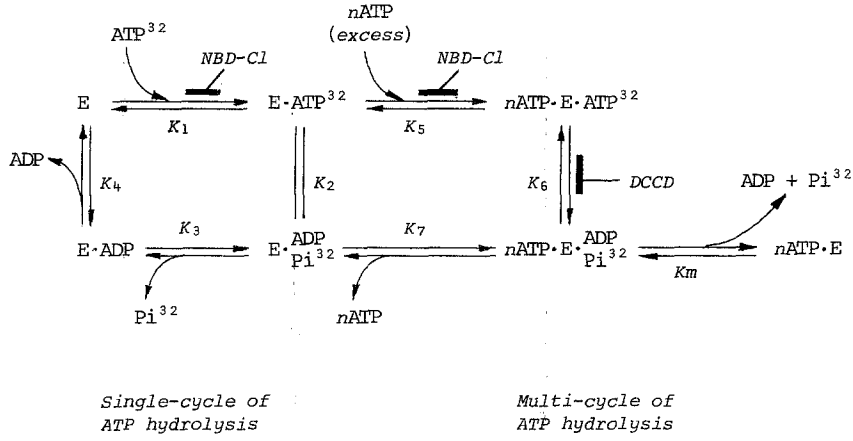

Fig. 2. Reaction mechanism of the vacuolar membrane H^+-ATPase. Elementary steps for single-cycle and multicycle ATP hydrolysis are shown. See text for details. The sites of inhibition by NBD-Cl and DCCD are indicated by the symbol ⊢. K_s (s = 1–7) are equilibrium constants and K_m is a Michaelis constant under the steady-state condition.

(2) The bound ATP, most of which is committed to hydrolysis, is split into ADP and Pi, and the products are slowly released from the enzyme. (3) The hydroltic step at the single catalytic site is stimulated in a positive cooperative manner by the presence of excess ATP (Fig. 2). It is noteworthy that the reaction mechanism proposed is similar to those for mitochondrial and bacterial F_0F_1-ATPases (Grubmeyer *et al.*, 1982; Cross *et al.*, 1982; Duncan and Senior, 1985; Futai *et al.*, 1988; Noumi *et al.*, 1986).

Interestingly, NBD-Cl inhibits enzyme activity under the two kinetic state equally, whereas DCCD inhibits only activity under multicycle ATP hydrolysis. It is also noted that NBD-Cl inactivates the catalytic site and results in inhibition of enzyme–ATP formation. DCCD does not affect the binding of ATP to a high-affinity catalytic site (see Fig. 2).

The membrane-bound H^+-ATPase on right-side-out vacuolar membrane vesicles is subjected to ATPase assay under two kinetic conditions. Here again, the single-cycle hydrolysis of ATP at the NBD-Cl sensitive catalytic site and its cooperative stimulation by excess ATP have been demonstrated (Hirata *et al.*, 1989). DCCD does not inhibit the activity of the single-cycle hydrolysis of ATP but inhibits strongly that of the multi-cycle hydrolysis of ATP as well as that of H^+-translocation across the vacuolar membrane (Uchida *et al.*, 1988a; Hirata *et al.*, 1989).

To address the question of the minimal molecular masses of the H^+-ATPase bound to vacuolar membranes for single-cycle and multi-cycle hydrolysis of ATP, radiation inactivation analysis has been carried out

(Hirata *et al.*, 1989). When vacuolar membrane vesicles are exposed to γ rays from ^{60}Co, the activities catalyzing the single-cycle and multicycle ATP hydrolysis both decrease as single exponential functions of the radiation dosage, and the susceptibility to irradiation of H^+-ATPase for the former reaction is 4-fold lower than that for the latter reaction. By applying the target theory, the functional molecular masses for single- and multicycle hydrolysis of ATP are determined to be $0.9–1.1 \times 10^5$ and $4.1–5.3 \times 10^5$ daltons, respectively. This result suggests that (1) the H^+-ATPase with a minimal composite of subunits *a* (67 kDa) and *b* (57 kDa), in which subunit *c* (20 kDa) is not necessarily involved operationally, can catalyze single-cycle hydrolysis of ATP in a DCCD-insensitive manner, and (2) for multicycle hydrolysis of ATP, the H^+-ATPase requires a properly organized oligomeric structure of $(a, b)_m c_n$, where $m \geqslant 3$, which directs a positive cooperative mechanism of ATP hydrolysis and coupled H^+-translocation in a DCCD-sensitive manner (Hirata *et al.*, 1989).

Cloning of the Structure Genes for Vacuolar Membrane H^+-ATPase Subunits

*c*DNA clones encoding the catalytic subunits (67 kDa) of the vacuolar membrane H^+-ATPases in *Neurospora* (Bowman *et al.*, 1988b) and in carrot (Zimniak *et al.*, 1988), and the regulatory subunits (57 kDa) of those in *Neurospora* (Bowman *et al.*, 1988a) and in *Arabidopsis* (Manolson *et al.*, 1988) have been isolated and their nucleotide sequences determined. The predicted 67-kDa subunits of the enzymes from *Neurospora* (M_r 67, 121) and carrot (M_r 68, 835) consist of 607 and 623 amino acid residues, respectively, and both are hydrophilic proteins with no obvious membrane-spanning regions. The carrot *c*DNA sequence exhibits high homology (62% identical) with the exons of the *Neurospora* genomic clone (Bowman *et al.*, 1988b; Zimniak *et al.*, 1988). In addition, both subunits show considerable homologies (over 30%) with the α and β subunits of F_0F_1-ATPases. The *Arabidopsis* *c*DNA, which encodes a hydrophilic, 57-kDa subunit composed of 492 amino acids, exhibits a high degree of sequence conservation with the *Neurospora* *c*DNA encoding the counterpart subunit (Manolson *et al.*, 1988; Bowman *et al.*, 1988a): There is a 73% amino acid sequence identity between the two subunits.

Recently, we have cloned the structure genes for a catalytic subunit *a* (*VMA1*)[3] and a DCCD-binding subunit *c* (*VMA3*)[4] of the H^+-ATPase in

[3]Hirata, R., Kawasaki, H., Nakano, A., Ohsumi, Y., Suzuki, K., and Anraku, Y., manuscript in preparation.

[4]Umemoto, N., Ohya, Y., and Anraku, Y., manuscript in preparation.

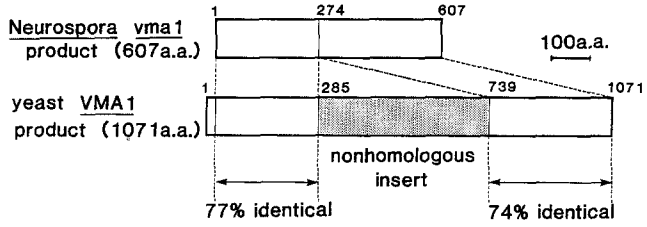

Fig. 3. Alignment of the yeast *VMA1* gene product with the *Neurospora VMA1* gene product. Open boxes represent highly homologous regions, and the hatched box shows the internal nonhomologous peptide insert.

S. cerevisiae and determined their nucleotide sequences (unpublished results). The partial amino acid sequence of the subunit *a* was determined from six tryptic fragments of the purified subunit. A 39-mer oligonucleoride probe, which corresponds to an oligopeptide IVAAVSPAGGDFK, was synthesized based on the codon usage index of yeast and used for screening of an yeast genomic DNA library that was constructed in our laboratory. Plasmid pRH151 thus isolated contains a single open reading frame encoding a polypeptide of 1071 amino acids (Fig. 3). The predicted amino acid sequence has been found to contain all the tryptic fragments and, when it was aligned with that of the catalytic subunit of *Neurospora* H^+-ATPase (Bowman *et al.*, 1988b), more than 75% of the amino acids are identical. From this and other results (unpublished observations), we designated this open reading frame as *VMA1* (vacuolar membrane H^+-ATPase).

As shown in Fig. 3[3], the predicted amino acid sequence of subunit *a* of the yeast vacuolar membrane H^+-ATPase, deduced from the nucleotide sequence of *VMA1*, shows the highest homology to the 67-kDa catalytic subunit of *Neurospora* enzyme (Bowman *et al.*, 1988b). More than 75% of the amino acid residues are identical over 590 amino acids, and when conservative replacements are taken into account, overall homology reaches about 95%. The subunit also shows high sequence homology to the catalytic subunits of carrot vacuolar membrane H^+-ATPase (62% identical; Zimniak *et al.*, 1988) and *Sulfolobus acidocaldarius* plasma membrane H^+-ATPase (48% identical; Denda *et al.*, 1988). In addition, the subunit *a* shows low but extensive sequence homology to the β-subunits of F_0F_1-ATPases. All these facts suggest existence of a common ancestral origin both in structural and functional motifs for a family of H^+-ATPases, and these motifs have evolved to establish the present-day H^+-translocating enzyme complexes including F-type (F_0F_1-H^+-ATPase), V-type (vacuo-lysosomal H^+-ATPase), and A-type ATPases (archaebacterial plasma membrane ATPase).

More exciting is the finding[3] that a predicted product of the *VMA1* gene contains an internal nonhomologous peptide insert of 454 amino acids (see

Fig. 3). This unique region must be removed during the gene expression and subunit biogenesis, since a matured subunit *a* in the vacuolar membrane has a molecular mass of 67 kDa and this insert contains no tryptic polypeptides so far identified. We have confirmed that there is no consensus sequence for splicing in the *VMA1* open reading frame, suggesting that a novel protein processing mechanism is involved in biogenesis of the subunit *a*.

Cloning of *VMA3*, the structure gene for subunit *c* of the yeast vacuolar membrane H^+-ATPase, has also been accomplished using a 43-mer oligonucleotide corresponding to the N-terminal amino acid sequence of the purified subunit[4]. The *VMA3* gene encodes a hydrophobic polypeptide of 160 amino acid residues, which shares extensive sequence homology of about 65% with the 16-kDa proteolipid from bovine chromaffin granules (Mandel *et al.*, 1988).

Disruption of the *VMA1* gene results in low spore viability and slower growth rate in nutrient-enriched medium, indicating that the function of subunit *a* is not essential for vegative growth[3]. A haploid strain containing a disrupted *VMA3* shows growth defect, the phenotype of which is similar to that of the haploid strain with the disrupted *VMA1*[4].

Most recently, Nelson *et al.*, (1989) cloned a conserved gene (or *VMA2*) encoding a 57-kDa subunit of the yeast vacuolar membrane H^+-ATPase and determined its nucleotide sequence. The predicted amino acid sequence shows extensive identity (over 70%) with the homologous subunits from *Arabidopsis* and *Neurospora* (Manolson *et al.*, 1988; Bowman *et al.*, 1988a).

Vacuole Is the Center for Regulation of Ionic Homeostasis

Current studies from our laboratory have demonstrated that yeast vacuoles are the metabolically active organelles and acidic compartments capable of regulating ionic homeostasis in the cytosol (Anraku, 1987a, b). Figure 4 shows that the H^+-ATPase functions as a primary H^+-pump and donates chemiosmotic energy for driving various secondary chemiosmotic solute pumps and ion channels.

There are seven nH^+/amino acid antiport systems including arginine, arginine-lysine, histidine, phenylalanine-tryptophan, tyrosine, glutamine-aspargine, and isoleucine-leucine uptake systems, which are all driven by Δp generated by the H^+-ATPase (Ohsumi and Anraku, 1981; Sato *et al.*, 1984a, b). These specific transport systems are shown to function in the formation of *in situ* differential compartmentation of cellular amino acids (Ohsumi *et al.*, 1988; Kitamoto *et al.*, 1988a, b). The vacuolar Ca^{2+} transport is also mediated in a similar way through a nH^+/Ca^{2+} antiport system (Ohsumi and Anraku, 1983), whose function is obligatorily required for regulation of free $[Ca^{2+}]$ in the cytosol (Anraku, 1987b).

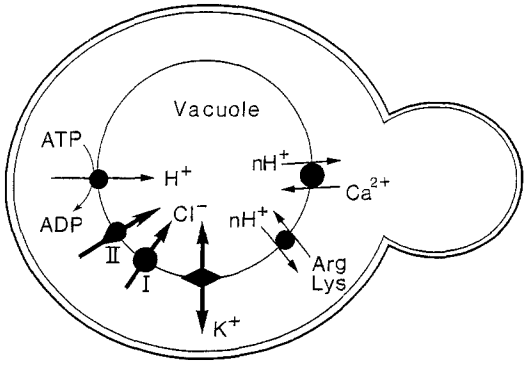

Fig. 4. Chemiosmotic work on the vacuolar membrane. The H^+-ATPase as a primary H^+-pump energizes the vacuolar membrane and donates Δp capable of driving various chemiosmotic solute pumps and channels. See text for explanation. Chloride transport systems I and II are DIDS-insensitive and DIDS-sensitive, respectively.

Recently, Wada *et al.*, (1986) studied the mechanism of acidification inside the vacuole, using isolated vacuolar membrane vesicles with right-side-out orientation. They found that the H^+-ATPase activity and total protonmotive force thereby generated are affected very little by Cl^-, but the anion elicits different responses for the formation of ΔpH and $\Delta\psi$ across the vacuolar membrane: (1) Cl^- stimulates the initial rate of H^+ uptake and the extent of ΔpH formation, whereas it reduces the extent of $\Delta\psi$ formation. (2) $^{36}Cl^-$ is actively taken up by the membrane vesicles in an ATP hydrolysis-dependent and CCCP-sensitive manner. These facts suggest that chloride transport across the energized vacuolar membrane is related to an acidification mechanism inside the vacuole, mainly through the interconversion of a $\Delta\psi$ into ΔpH, leaving the total protonmotive force unchanged (Wada *et al.*, 1986). The Cl^--dependent H^+ uptake or acidification process has been found to be mediated by two independent components: one is a saturable component with an apparent K_m value for Cl^- of 20 mM and is DIDS-insensitive, and the other is a linear component and is inhibited effectively by DIDS, a potent inhibitor for anion transport systems (see Fig. 4).

In this context of chemiosmotic energy conversion, the function of a vacuolar cation channel, which has been characterized recently (Wada *et al.*, 1987; Tanifuji *et al.*, 1988), is worth mentioning. This cation channel, whose characteristic features are mainly studied by an electrophysiological method using artificial planar bilayer membranes incorporated with isolated vacuolar membrane vesicles, shows the following unique properties: (1) It conducts K^+ and shows a large single-channel conductance of about 435 S in 0.3 M KCl. (2) It has, however, broad ion selectivity and can conduct other cations such as Cs^+, Na^+, Li^+ but does not conduct Cl^-. (3) The opening of

this channel is regulated by the membrane potential, the optimal potential being at around 30 mV, interior positive, and with the presence of Ca^{2+} on the cytoplasmic side. All these physiological features of the K^+-channel (see Fig. 4) are consistent with the idea that it functions as an essential component, or a chemiosmotic energy converter operationally coupled with the H^+-ATPase, Cl^- transport systems and other solute transport systems, for the formation and regulation of the chemical and electrical potential differences across the vacuolar membrane.

Conclusion and Perspectives

The H^+-ATPase of the vacuolar membrane of *S. cerevisiae* is the third type of H^+-pumping ATPase in the microorganism. A family of this type of H^+-ATPase is now known to be distributed in a wide variety of eukaryotic vacuolysosomal organelles and archaebacteria. The enzyme confers to the vacuole an autonomous ability for membrane energization, which causes hierarchical energy conversion and the transformation required for chemiosmotic work under functional cooperation with other secondary chemiosmotic solute pumps and channels residing on the membrane. The biochemical, molecular-biological, and physiological aspects of these H^+-ATPases will provide further insights into the bioenergetics and regulation of cellular ion homeostasis and growth control in eukaryotic cell systems.

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