

Review

Vacuolar H⁺-pyrophosphatase

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Abstract

The H⁺-translocating inorganic pyrophosphatase (H⁺-PPase) is a unique, electrogenic proton pump distributed among most land plants, but only some alga, protozoa, bacteria, and archaeobacteria. This enzyme is a fine model for research on the coupling mechanism between the pyrophosphate hydrolysis and the active proton transport, since the enzyme consists of a single polypeptide with a calculated molecular mass of 71–80 kDa and its substrate is also simple. Cloning of the H⁺-PPase genes from several organisms has revealed the conserved regions that may be the catalytic site and/or participate in the enzymatic function. The primary sequences are reviewed with reference to biochemical properties of the enzyme, such as the requirement of Mg²⁺ and K⁺. In plant cells, H⁺-PPase coexists with H⁺-ATPase in a single vacuolar membrane. The physiological significance and the regulation of the gene expression of H⁺-PPase are also reviewed. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: cDNA; H⁺-Pyrophosphatase; Proton pump; Vacuolar membrane; (Plant)

1. Introduction

The H⁺-translocating inorganic pyrophosphatase (H⁺-PPase) is an electrogenic proton pump that acidifies vacuoles in plant cells [1]. H⁺-PPase is a unique proton pump with the following three char-

acteristics. First, it consists of a single polypeptide with a molecular mass of about 80 kDa. Second, the enzyme utilizes a simple, low-cost substrate pyrophosphate (PPi) that has a high-energy phosphoanhydride bond. PPi is generated as a by-product of several biosynthetic processes for macromolecules, such as protein, RNA and cellulose. Third, this efficient proton pump coexists with H⁺-ATPase in a single vacuolar membrane in plant cells [2]. This property is related to the physiological function of H⁺-PPase in plant cells. With respect to the first and second properties, H⁺-PPase is a fine model for studying the coupling mechanism between the hydrolysis of a high-energy phosphate bond and the translocation of protons.

Soluble PPases of prokaryotes and eukaryotes have been shown to form a large family of homologous enzymes. Although the cytoplasmic PPase has not yet been purified from plants, a soluble PPase

Abbreviations: CAM, Crassulacean acid metabolism; CS, conserved segment; DCCD, *N,N'*-dicyclohexylcarbodiimide; H⁺-PPase, H⁺-translocating inorganic pyrophosphatase; HPLC, high-performance liquid chromatography; NCD, *N*-cyclohexyl-*N'*-[4-(dimethyl amino)- α -naphthyl]carbodiimide; PPi, inorganic pyrophosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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gene has been cloned from *Arabidopsis* [3]. In addition to the well-known soluble PPases, membrane-bound PPases have recently been discovered in the plant thylakoid membrane [4] and plant mitochondria [5]. These membrane-bound PPases, however, did not show proton pump activity. For the present, PPases in a wide variety of organisms can be divided into three classes; namely, soluble PPase, membrane-associated PPase, and H^+ -PPases. Only H^+ -PPase among the three types of PPase has the ability to transport protons across the membrane. The overall structure of the membrane-associated PPases is unclear, although their catalytic subunits have been isolated from several organisms as shown in Table 1. Among the three classes of PPase, the soluble PPase has been well characterized at the molecular level. Unexpectedly, the primary structure of H^+ -PPase is not homologous to the soluble PPase except for the small domain of the catalytic site [13]. The tertiary structure and kinetic properties of the soluble PPase have been reviewed elsewhere [8]. The substrate for H^+ -PPase, PPi, is produced as a by-product of several metabolic processes, such as polymerization of DNA and RNA, and syntheses of aminoacyl-tRNA (protein synthesis), ADP-glucose (starch synthesis), UDP-glucose (cellulose synthesis) and fatty acyl-CoA (β -oxidation of fatty acid) in plant cells (Fig. 1).

2. Enzymatic properties of the H^+ -PPase

2.1. Purification

The activity of a membrane-associated PPase was first reported for the crude membrane fraction prepared from plants in the mid-1970s, and then H^+ -PPase was defined to be located in vacuolar membranes in the first half of the 1980s (for review see [1]). Several groups had attempted to find the enzyme protein for several years. The vacuolar H^+ -ATPase was the first to be isolated (for review see [14]). Then three papers concerning the purification of vacuolar H^+ -PPase were published in 1989 [15–17]. The use of the young plant tissues, such as seedling hypocotyls, was a key point in obtaining sufficient amount of H^+ -PPase, because the mature tissues of most plants have a low level of the enzyme. H^+ -PPase could be solubilized from vacuolar membranes by 0.4% lyso-phosphatidylcholine [15], 50 mM CHAPS [18], 2–2.5% Triton X-100 [16,17], or 40 mM octylglucoside [19]. Then the enzyme was isolated from the solubilized fraction by anion exchange column chromatography and gel filtration. In contrast to vacuolar H^+ -ATPase, the purified H^+ -PPase consisted of a single polypeptide of 67 or 73 kDa. The single-polypeptide composition of H^+ -PPase has been proposed from

Table 1
Classification of inorganic pyrophosphatases

PPase	Subunit mass (kDa)	Amino acid number ^a	Ref.
H^+ -PPase (PPi synthase)			
Plant (vacuole)	80 ^b	761–771	[1] ^c
<i>Rhodospirillum rubrum</i> (chromophore)	67.5 ^b	600	[6,7]
Soluble PPase			
<i>Escherichia coli</i>	20 ^b	175	[8]
<i>Saccharomyces cerevisiae</i>	32.5 ^b	286	[8]
<i>Arabidopsis thaliana</i>	30 ^b	263	[3]
Potato	24, 25 ^b	211, 217	[9]
Membrane-associated PPase			
Spinach (chloroplast thylakoid)	55		[4]
<i>Saccharomyces cerevisiae</i> (mitochondria)	32 ^b	310	[10]
<i>Sulfolobus acidocaldarius</i> (plasma membrane)	17–18		[11]
Pea (mitochondria)	35		[5]
<i>Syntrophus gentianae</i>	Unknown		[12]

^aNumber of amino acid residues of the polypeptide deduced from the cloned DNA.

^bMolecular mass calculated from the cDNA.

^cSee text.

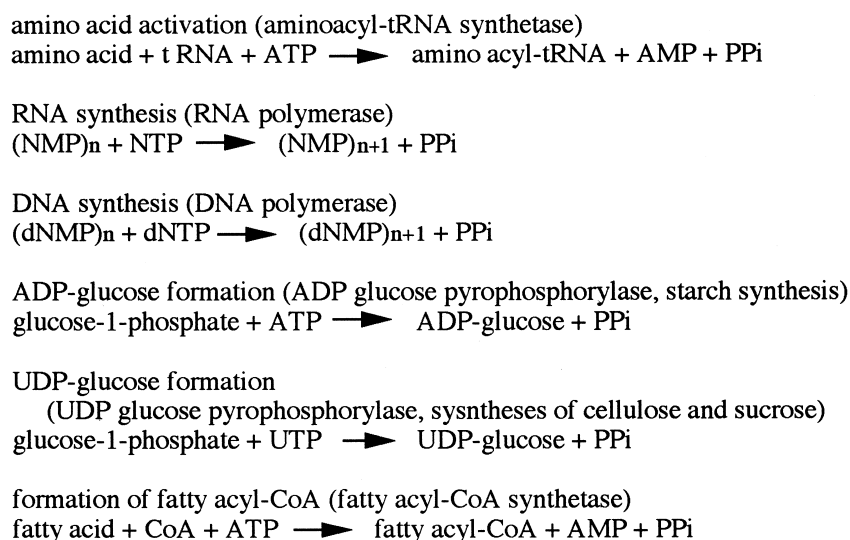


Fig. 1. Main steps of PPi production in plant cells.

an SDS-PAGE profile of the purified preparation and then confirmed by reconstitution into proteoliposome with the purified polypeptide [18,20].

2.2. V_{max} and K_m

The actual free energy change for PPi hydrolysis in the cytoplasm has been calculated to be 27.3 kJ/mol at pH 7.3 [21]. The H⁺/PPi stoichiometry of H⁺-PPase has been determined to be 1, and the steady-state pH generated by the enzyme 3.2 [22]. Specific activities of H⁺-PPase in vacuolar membranes vary with the plant species, tissues, and assay conditions. Typical values are 1.10, 0.30, 0.52, 0.35, 1.56, and 0.22–0.71 μmol PPi/min per mg of membrane protein for the seedling hypocotyl of mung bean [15], storage tissue of red beet [16,17], *Arabidopsis* leaf [23], cotyledon of pumpkin seedling [24], *Acetabularia* [25], and CAM plants [19], respectively. The actual substrate for H⁺-PPase is a Mg²⁺-PPi complex (Mg₂PPi) [1,26–28]. The purified enzyme requires phospholipid for catalysis [15–17]. The specific activities of PPi hydrolysis by the purified H⁺-PPases of mung bean and red beet were 8.5 and 3.0 μmol/min per mg, respectively. The enzyme purified from CAM plants also has shown similar values [19]. A high specific activity of 20 μmol/min per mg has been reported for the purified preparation of *Rhodospirillum rubrum* H⁺-PPase [6]. The half-maximum activity of the purified enzyme was obtained at 35 μM

MgPPi in the presence of 1 mM Mg²⁺ in the assay medium. H⁺-PPase in the purified form and in the vacuolar membrane expresses its maximal velocity at more than 200 μM PPi in the presence of 1 mM MgSO₄. From kinetic analysis, the measured K_m for the substrate has been reported to be 130 μM [29], 2–5 μM [27], and 2 μM [26]. The reported value of the cytosolic PPi concentration of approximately 200 μM is enough for expression of maximal activity of H⁺-PPase in plant cells.

2.3. Mg²⁺ and K⁺ as cofactors

H⁺-PPase requires free Mg²⁺ as an essential cofactor. MgCl₂ or MgSO₄ was added to the buffers for solubilization and purification of the enzyme at 1–2 mM [15,17,29,30]. Binding of Mg²⁺ stabilizes and activates the enzyme. The apparent K_m values for Mg²⁺ have been reported to be 42 μM [29] and 20–23 μM [26]. However, the exact number of Mg²⁺-binding sites on H⁺-PPase is unclear. Baykov et al. [27] have reported the presence of high-affinity (K_m = 23–31 μM) and low-affinity (K_m = 0.25–0.46 mM) Mg²⁺-binding sites of mung bean H⁺-PPase. H⁺-PPase, like the yeast cytosolic PPase, has been proposed to have two different Mg²⁺-binding sites in a single enzyme molecule. The cytosolic free Mg²⁺ concentration has been determined to be about 0.4 mM [31], and under these conditions H⁺-PPase expresses more than 90% of its full activity. Binding

of Mg^{2+} to H^+ -PPase not only activates the enzyme but also protects it from heat inactivation [26].

Potassium ion is also an essential cofactor of H^+ -PPase. K^+ stimulates H^+ -PPase more than 3-fold in most cases. Thus, the PPI-dependent, K^+ -stimulated, and F^- -insensitive activity is defined as that of the vacuolar H^+ -PPase. The K_m value for K^+ stimulation has been reported to be 1.27 mM [32]. The maximal activity could be obtained in the presence of more than 30 mM KCl in most cases. H^+ -PPase in *Vicia faba* guard cells, however, requires a high concentration of K^+ ($K_m = 51$ mM) [33]. Gordon-Weeks et al. [32] reported that Tris at more than 25 mM inhibited this activation of H^+ -PPase by K^+ , and the inhibitory effect of Tris, and Bis-Tris-propane, was marked at KCl concentrations less than 10 mM. At present, the biochemical mechanism of competitive inhibition of K^+ activation by Tris and other pH buffers remains to be resolved. In some cases, K_2SO_4 gave higher activities than KCl for hydrolysis of the purified enzyme [15,17] and the PPI-dependent H^+ transport activity of vacuolar membranes [34].

It has been a matter of argument whether or not H^+ -PPase transports K^+ into the vacuole. Davies et al. [35] has proposed from patch clamp studies of red beet vacuoles that the H^+ -PPase functions as a H^+/K^+ symporter with a coupling ratio of 1.3 $\text{H}^+ : 1.7 \text{K}^+ : 1 \text{PPI}$ [35]. Obermeyer et al. [36] also analyzed vacuoles of *Chenopodium rubrum* by the patch clamp technique, and obtained evidence for the possible role of the H^+ -PPase in K^+ transport. This is a very interesting, attractive proposal, but we have not yet reached to a consensus. There is counter evidence against this proposal. Experiments with reconstituted H^+ -PPase into proteoliposome and $^{42}\text{K}^+$ [20] failed to confirm the K^+ transport. Ros et al. [37] also found that no active transport of K^+ by H^+ -PPase was detectable by fluorescent probe measurements. Gordon-Weeks et al. [32] suggested the need to re-examine the K^+ transport assay using a pH buffer that does not affect K^+ stimulation.

2.4. A possible new system for electrochemical analysis

Recently, Yabe and his colleagues established a method for preparation of giant protoplasts with giant vacuole-type structures from normal *Escheri-*

chia coli cells [38]. If this useful system can be applied to yeast cells, we will be able to determine the PPI-dependent K^+ current of a giant vacuole with or without H^+ -PPase, since it is now possible to express the plant H^+ -PPase gene heterologously in yeast cells [39–41]. Reconstituted liposomes are not large enough to allow measurement of the electric current generated by an ion pump such as H^+ -PPase. This new giant vacuole system is expected to provide more clear information about the PPI-dependent K^+ current.

It must be resolved whether or not the pH gradient across vacuolar membranes generated by H^+ -ATPase drives a reversal reaction of H^+ -PPase (formation of PPI from Pi). In *R. rubrum*, H^+ -PPase (PPI synthase) can both synthesize and hydrolyze PPI under physiological conditions [42]. The reversal reaction of H^+ -PPase has been considered previously [22,43,44], and recently Façanha and Meis [45] demonstrated that ^{32}Pi was incorporated into PPI through maize H^+ -PPase. It was indicated by isotope exchange rather than net synthesis of PPI. The patch clamp technique using a giant vacuole that contains H^+ -PPase may provide us with more conclusive information about the reversibility of the catalytic cycle of plant vacuolar H^+ -PPase.

2.5. Regulation of enzyme activity

Vacuolar H^+ -PPase has been reported to be reversibly inhibited by Ca^{2+} through formation of CaPPI which is a strong, competitive inhibitor as for the soluble PPases [8], and the approximate K_i for CaPPI is 17 μM [29]. Rea et al. [46], however, proposed that the free Ca^{2+} ion, rather than CaPPI , is the inhibitory ligand of H^+ -PPase. They pointed out that Ca^{2+} is a possible modulator of H^+ -PPase in plant cells. Also, the concentration of cytosolic Mg^{2+} is a possible element for fine regulation of H^+ -PPase activity in plant cells as proposed previously [27,28]. The negative regulation of H^+ -PPase activity through changes in cytosolic free Ca^{2+} has been discussed for bean guard cells [33] and barley aleurone cells [47]. The inhibitory effect of Na^+ on H^+ -PPase has been reported for vacuolar membranes of red beet [48].

Under physiological conditions, Fishcher-Schliebs et al. [49] have observed H^+ -PPase-activated ATP-

dependent H^+ -transport in vacuolar membrane vesicles of *Kalanchoë*. The initial rate of ATP-dependent H^+ -transport was stimulated when the membrane vesicles were pre-energized by H^+ -PPase, although pre-energization by H^+ -ATPase did not affect the H^+ -PPase. They proposed that the H^+ -ATPase modulation by H^+ -PPase might be due to the interaction between H^+ -PPase and the subunit A of H^+ -ATPase. Inhibition of H^+ -ATPase inhibits the proton pump activity of H^+ -PPase in vacuolar membranes of *Acer pseudoplatanus* [50].

Among the artificial substances tested, Zhen et al. [51] reported that aminomethylenebisphosphonate is a potent inhibitor of H^+ -PPase, and its apparent inhibition constant for mung bean H^+ -PPase is 1.8 μ M. The reagent inhibits both types of plant vacuolar H^+ -PPases and *R. rubrum* PPi synthase, but not V-ATPase or cytosolic soluble PPases of rat liver and *Saccharomyces cerevisiae*. They commented that aminomethylenediphosphonate, as a type-specific inhibitor of vacuolar type H^+ -PPase, is not restricted to plant systems but also extends to nonplant cells containing H^+ -PPase homologues [52]. Recently, Gordon-Weeks et al. [53] examined the effectiveness of bisphosphonates from the structural aspects, and they reported that a nitrogen atom in the carbon chain of bisphosphonates increased the inhibitory effect. If a specific photo-affinity reagent will be synthesized as a derivative of bisphosphonates, it will become a useful tool to identify the catalytic pocket of this enzyme.

3. Molecular structure of H^+ -PPase

3.1. Tertiary structure

H^+ -PPase has been strongly suggested to exist as a dimer of identical subunits of 80 kDa. Radiation-inactivation analysis has yielded a functional mass of 160 kDa for PPi hydrolysis [54]. For the pumpkin enzyme, the radiation-inactivation size has been reported to be 139 kDa. Tzeng et al. [55] have also reported similar values of 141 and 158 kDa for PPi hydrolysis and PPi-dependent H^+ translocation, respectively. The native molecular size of H^+ -PPase has been determined to be 135 kDa in gel permeation HPLC [56]. Sarafian et al. [57] reported that the radiation-inactivation size for PPi-dependent H^+ trans-

port was about 446 kDa. SDS-PAGE after cross-linking of the purified H^+ -PPase with dimethyl suberimidate showed a band of 158 kDa [58]. In native PAGE, the purified H^+ -PPase migrated as a detergent-enzyme complex of 480 kDa in the presence of Triton X-100 [58]. It is clear that a single catalytic subunit is insufficient for H^+ transport activity; however, the exact degree of oligomerization of H^+ -PPase in the vacuolar membrane remains to be determined directly.

3.2. Molecular cloning

The first cDNA for H^+ -PPase has been cloned from *Arabidopsis* (database accession no. M81892) [59] by immunological screening of an expression library with the antibody to mung bean enzyme. Then the cDNAs have been cloned from various land plants, such as barley (D13472) [60], red beet (L32791, L32792) [61], tobacco (X77915, X83730, X83729) [62], rice (D45383, D45384) [63], mung bean (AB009077) [64], and pumpkin (D86306) [65]. H^+ -PPases of these plant species have been reported to consist of 761–771 amino acids with a *pI* of approximately 5.0 (Table 1). Their calculated molecular masses range from 79 841 to 80 800 Da. The amino acid sequences are highly conserved among land plants with 86 to 91% identity. The least conserved region is the amino-terminal part (the first 60 residues). From a comparison of these sequences, it is hard to estimate the essential regions for enzymatic function and binding sites of metal ions such as K^+ and Mg^{2+} .

Recently, the primary structures of H^+ -PPases have been reported for *R. rubrum* (PPi synthase) (database accession no. AF044912) [7,66], a marine alga *Acetabularia acetabulum* (D88820) [67], and green alga *Chara corallina* (AB018529) [68]. The overall identities of amino acid sequences of H^+ -PPase among these three phylogenically separated organisms were low (35–46%). The identity of *R. rubrum* PPase synthase (660 residues) is 36–39% with vacuolar H^+ -PPases of land plants, and 40% with *A. acetabulum* H^+ -PPase. *A. acetabulum* H^+ -PPase (721 residues) is about 47% identical with land plant H^+ -PPases. A high identity of about 71% was obtained for H^+ -PPases between *C. corallina* (793 residues) and land plants.

Fig. 3. Topological model of H⁺-PPase from mung bean. Fourteen transmembrane domains were predicted from the hydropathy profile (TMPred program). In our model, the putative substrate-binding site (253–263) is in cytosolic loop *e*. The *N*-ethylmaleimide reactive Cys-630 demonstrated previously for Arabidopsis enzyme [69,70]. Three conserved segments are marked (CS1, CS2 and CS3).

There is evidence suggesting that CS1 is a cytoplasmic loop, although the site of CS2 remains to be determined. Takasu et al. [69] prepared an antibody specific to a peptide which corresponds to the catalytic site motif (DVGADLVGKVE), and reported that this antibody suppressed the PPI-dependent proton-pump activity of the vacuolar membrane vesicles. Recently, Sakakibara et al. [70] revealed that the nucleotide sequences encoding CS1, CS2, and CS3 are each interrupted by a single exon. Therefore, these conserved segments are expected to be divided into more detailed, short domains.

3.4. Functional residues

Yang et al. [71] examined the effect of chemical modification of Tyr residues in H⁺-PPase on the enzymatic activity. They found a substrate-protectable Tyr residue; however, the tetranitromethane-reactive Tyr residue has not yet been identified among 22 Tyr residues. Gordon-Weeks et al. [26] performed a systematic analysis of amino acid residues of H⁺-PPase to determine the binding constants for the substrate and Mg²⁺ using covalent inhibitors, such as 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBDCl) and phenylglyoxal. Kim et al. have clearly demonstrated that substrate-protectable *N*-ethylmaleimide (NEM) inhibition is due to NEM binding to Cys-634 of *Arabidopsis* enzyme [72,73]. This result has been confirmed for pumpkin enzyme [65]. This Cys residue is conserved in H⁺-PPases of all species including *Chara*, *Acetabularia*, and *Rhodospirillum rubrum*.

As described above, a sequence DVGADLVGKVE of H⁺-PPase has been proposed to participate directly in substrate and Mg²⁺ binding, based on the three-dimensional structure of soluble PPases [1,8,74]. The inhibitory effect of the antibody specific to this sequence also confirms this proposal [69]. By a mutational analysis of DNA for mung bean H⁺-PPase expressed in yeast, three charged residues in this common sequence (Asp-253, Lys-261, and Glu-263) have been demonstrated to be essential for enzymatic activity [41]. The importance of this common sequence is evident. However, some other sequence may be essential to form the catalytic site of H⁺-PPase. Recently, crystal structures of *E. coli* and yeast soluble PPases have been published (for review

see [8]). It has been demonstrated that the core structure of soluble PPase consists of eight β -strands and two α -helices [8,75,76]. The active site cavity is formed with several charged residues, which are located on different parts of these β -strands. The active site of H⁺-PPase is also estimated to be composed of several cytoplasmic loops. We have to identify the counterparts of the common sequence DVGADLVGKVE in the three-dimensional structure.

N,N'-Dicyclohexylcarbodiimide (DCCD) is known as a potent blocker of proton conductance of F₀F₁-ATPase. DCCD is reactive with carboxyl groups in hydrophobic regions of proteins. The purified H⁺-PPases have been reported to be covalently labeled by [¹⁴C]DCCD [15,77] and inhibited by DCCD. The DCCD-binding site may be a key residue to determine the coupling mechanism between PPI hydrolysis and H⁺-translocation. Zhen et al. [52] have proposed the DCCD-binding residues (Glu-305 and Asp-504 of *Arabidopsis* enzyme) by the method of site-directed mutagenesis. They paid attention to eight acidic amino acid residues (Glu-119, Glu-229, Glu-305, Glu-427, Asp-504, Asp-573, Glu-667, and Glu-751) which were estimated to be located near or within transmembrane domains of the enzyme. The replacement of the six acidic residues, except for Glu-305 and Asp-504, with their corresponding amides had no effect on inhibition by DCCD [52]. The Glu-305 and Asp-504 residues are located at the cytoplasmic side in the transmembrane domains in their membrane topology model. The substitution of Glu-305 (E305Q and E305D) and Asp-504 (D504N and D504E) resulted in loss of both PPI hydrolysis and H⁺ pump activities. Thus, it has been concluded that the Glu-305 and Glu-504 directly participate in DCCD binding and are critical for catalysis [52].

In pumpkin H⁺-PPase, however, Maruyama et al. [65] have identified the carbodiimide-reactive Glu residue (Glu-749) (Glu-751 of *Arabidopsis* H⁺-PPase) which is located at the last C-terminal transmembrane domain by using a fluorescent inhibitor, *N*-cyclohexyl-*N'*-[4-(dimethyl amino)- α -naphthyl]carbodiimide (NCD). The inhibition of H⁺-PPase by NCD was protected by 2 mM Mg²⁺. They proposed that the DCCD- (or NCD-) binding site is near by the Mg²⁺-binding site. The Glu residue corresponding to Glu-749 of pumpkin H⁺-PPase is conserved among

all H^+ -PPases except for *Rhodospirillum* enzyme. At present, it cannot be concluded whether H^+ -PPase contains two or three inhibitory DCCD-binding sites. It must be noted that the reactivity of DCCD with H^+ -PPase may be markedly changed by the concentration of Mg^{2+} as mentioned previously [52,65].

Also, the mutagenic experiments revealed the importance of the Glu-427 in enzymatic activity [52]. *Arabidopsis* H^+ -PPase in which the Glu-427 residue was substituted to Gln was insensitive to DCCD. Interestingly, this substitution mutant gave a low PPI-dependent H^+ -translocation activity, although the PPI hydrolysis activity remained (50% of the wild-type enzyme). The Glu-427 in the ninth transmembrane domain faces the cytosol in their model and has been proposed to be located at the coupling site of H^+ -PPase. The Glu residue corresponding to Glu-427 of *Arabidopsis* H^+ -PPase is conserved among H^+ -PPase of all species including *Acetabularia* and *Rhodospirillum*. It has been speculated that Glu-427 may be the first H^+ -carrying residues of H^+ -PPase to transport H^+ from the cytosol into the vacuole [52].

4. Regulation of gene expression and activity of H^+ -PPase

4.1. Cell growth and H^+ -PPase

A relatively high content and activity of H^+ -PPase in vacuolar membranes of growing tissue on the basis of fresh weight has been reported for mung bean in comparison with the mature part of seedling hypocotyls [78]. It has been confirmed to be due to active transcription of the H^+ -PPase gene [64]. The growing part of the hypocotyl is composed of relatively small cells with small central vacuoles. The relative content of H^+ -PPase on the basis of membrane protein in these young cells is slightly higher than that of mature grown cells. It has been concluded that the relative distribution density of H^+ -PPase with respect to the surface area of vacuolar membrane did not change during tissue elongation, although the size of vacuoles in young cells is less than 1% of that in mature cells. H^+ -PPase in growing cells functions to maintain sufficient osmoticum to

compensate for dilution effects resulting from the influx of water.

Smart et al. [79] have investigated the changes in the key enzymes involved in the development of cotton fibers after anthesis. Fiber cells are single-celled trichomes, and the cells elongate at peak rates in excess of 2 mm/day. The H^+ -PPase gene is constantly transcribed, and the H^+ -PPase activity changed dramatically during cell elongation with a peak at a few days after the peak rate of fiber elongation [79]. The increased level of H^+ -PPase has been thought to support the acidification of expanding vacuoles and the secondary active transport systems utilizing a proton motive force. Also, Lerchl et al. [62] reported the occurrence of changes in the mRNA levels of H^+ -PPase during leaf development of tobacco with respect to conversion from a sink to a source organ. All steady-state mRNA levels of three isoforms of H^+ -PPase were high in young sink leaves, but they decreased during leaf maturation. Furthermore, they have reported the daily rhythm of H^+ -PPase mRNA accumulation with a minimum at high noon and a severalfold increased signal at midnight [62]. High levels of H^+ -PPase activity and the subunit were also observed in young fruit tissue of pear [80].

In the pear fruit [80] and mung bean hypocotyl [78], the H^+ -PPase activity is several times higher than the V-ATPase activity. H^+ -PPase is the main proton pump of vacuolar membranes in most young tissues. In contrast to the H^+ -PPase level which decreases during tissue development, the V-ATPase level was relatively constant during growth and maturation. As a result V-ATPase became a major proton pump of vacuolar membranes in mature tissues.

The seedling hypocotyl is a typical example of young growing tissues. Some properties of H^+ -PPase of the mung bean hypocotyl are compared with V-ATPase in Table 2. The activities of H^+ -PPase and V-ATPase in the vacuolar membrane fraction of mung bean hypocotyls were 1.10 and 0.40 $\mu\text{mol PPI/min per mg}$, respectively [15,81]. There is no marked difference in the molecular activity between H^+ -PPase and V-ATPase. The high activity of H^+ -PPase is due to a high content of the enzyme (0.57 nmol/mg of protein). In this connection, the content of vacuolar aquaporin as a tetramer has been calculated to be about 3–4 nmol/mg of vacuolar membrane protein [82].

Table 2
Comparison of H^+ -PPase and V-ATPase in mung bean hypocotyl

Proton pump	Molecular size (kDa)	Molecular activity (s^{-1})	Content in vacuolar membranes (nmol/mg of protein)
H^+ -PPase	80 ^a	33 ^b	0.57 ^c
V-ATPase	647 ^d	45 ^e	0.15 ^e

The values were calculated for H^+ -PPase [15] and V-ATPase [81] in vacuolar membranes of hypocotyl prepared from 3.5-day-old mung bean seedlings.

^aValue calculated from cDNA [64]. H^+ -PPase is thought to exist as a homodimer of 80 kDa protein.

^bValue per dimer.

^cValue for dimeric form.

^dValue calculated from sizes of nine subunits with assumption that there are three copies of 68- and 57-kDa subunits and six copies of 16-kDa subunit in an enzyme complex.

^{ee}Value per whole enzyme.

The high content of H^+ -PPase is reasonable from the perspective of the cell's energetics. In growing tissue, RNAs, proteins and cellulose are actively synthesized for construction of cells and, as a result, a large amount of PPi is produced as a by-product of these metabolic processes as shown in Fig. 4. PPi accumulation in the cytosol to high concentrations inhibits these polymerization reactions. The vacuolar H^+ -PPase scavenges the PPi in the cytosol and uses it as a source of energy for active transport of protons into the expanding vacuoles. In mature cells, metabolic activity decreases and PPi may not be available in such large amounts. In addition, the rate of transport of solutes into the vacuole also decreases and

expansion of the vacuole ceases. Indeed, H^+ -PPase activity has been reported to be lower than that of V-ATPase in mature tissues [80,83].

4.2. Changes in H^+ -PPase level under stress conditions

The level of H^+ -PPase in plants is regulated under stress conditions. Colombo and Cerana [84] have reported an increment of H^+ -PPase activity in carrot suspension cells with NaCl treatment. Kasai et al. [85] have examined the effect of mineral nutrients, such as K^+ , NO_3^- , and Ca^{2+} , on H^+ -PPase in rye roots. Both PPi hydrolysis and PPi-dependent proton pumping activities in the plants grown under mineral-deficient conditions were three times greater than those in plants grown under normal conditions. The increased PPi-hydrolysis activity in the vacuolar membrane was $0.14 \mu\text{mol/min per mg}$. Since there was no difference in the amount of H^+ -PPase protein, it was suggested that there is an activation of H^+ -PPase in the rye grown under nutrient stress conditions, and that a high activity of H^+ -PPase resulted in the marked reduction of PPi level in roots grown in mineral-deficient medium. They suggested a possibility that Ca^{2+} or cytokinin may modulate the H^+ -PPase activity.

Rea and Poole [1] pointed out the importance of H^+ -PPase in plant cells under anoxia and cold stress. The following two reports clearly support their proposal. Carystinos et al. [86] reported that the relative levels of transcript and enzyme activity of H^+ -PPase increased notably under anoxia and chilling (10°C) in seedlings of rice, an anoxia-tolerant species. They

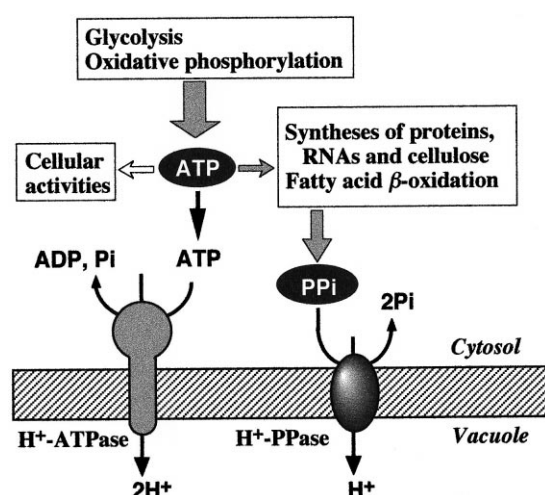


Fig. 4. PPi production and vacuolar membrane H^+ -PPase and H^+ -ATPase. PPi is supplied as a by-product of biosyntheses of macromolecules such as RNAs, proteins, and cellulose and β -oxidation of fatty acids.

observed a 75-fold increase in the enzyme activity from 0.0133 to 1.0 $\mu\text{mol}/\text{min}$ per mg of vacuolar membrane protein after 6 days of anoxia. The enzyme amount decreased to the original level after return to air. They proposed that the induced H^+ -PPase may replace V-ATPase under energy stress to maintain the vacuole acidity. The activity of H^+ -PPase activity, but not V-ATPase, increased approximately 1.5- to 2-fold in mung bean hypocotyls under low-temperature stress at 4°C [87]. This increment has been thought to be due to a shift toward fermentative metabolism in hypocotyl cells, because the rate of ATP generation decreased. On the other hand, the H^+ -PPase activity slightly decreased and the V-ATPase markedly decreased in mung bean hypocotyls after chilling at 0°C [88]. Under these severe conditions, plants that are sensitive to low temperatures suffer serious chilling injuries [89].

In some plants such as *Mesembryanthemum crystallinum*, the transition of the metabolic state from C_3 photosynthesis to crassulacean acid metabolism (CAM) takes place by treatment with NaCl. During C_3 -CAM transition, the V-ATPase activity markedly increased [90]. It has been demonstrated to be due to de novo synthesis of V-ATPase. In contrast, the H^+ -PPase activity was maximal in the preparations of young plants and decreased after the induction of CAM by NaCl treatment. Thus, V-ATPase is the main vacuolar proton pump in the CAM state. The vacuolar proton pump is discussed in detail in this issue.

4.3. H^+ -PPase in germinating seeds

Seed germination is one of the most drastic physiological changes in plant cells. Protein-storage vacuoles in dry seeds are transformed to central (or vegetative) vacuoles after seed germination. Protein-storage vacuoles do not contain H^+ -PPase or V-ATPase. During transformation to vacuoles in pumpkin cotyledons, both vacuolar proton pumps were synthesized actively and accumulated to relatively high levels comparative to vegetative tissue [24]. The PPI-dependent proton pump activity was 4-fold greater than that of V-ATPase. An acidic condition maintained by the proton pumps may be essential to the hydrolytic enzymes in vacuoles. The membrane proteins in the protein-storage vacuoles

were substituted with those of vacuolar membranes in germinating seeds. At this step, the seed-specific vacuolar aquaporin α -TIP was thoroughly replaced by γ -TIP (VM23) in pumpkin cotyledons [24]. It is unclear whether or not the seed-specific H^+ -PPase exists in plants. During senescence of cotyledons after 2 weeks of sowing, both H^+ -PPase and V-ATPase activities decreased to low levels [91]. Furthermore, H^+ -PPase has been detected together with V-ATPase in the protein storage vacuoles of pea cotyledon in a long term from early to mature stages of seed maturation [92].

The presence of H^+ -PPase and V-ATPase has been confirmed in vacuoles of germinating barley aleurone [47]. Incubation of aleurone protoplasts for 20 h in 5 μM gibberellic acid caused the vacuolar pH to fall from 6.6 to 5.9, although abscisic acid had no effect. Acidification of aleurone vacuoles by proton pumps facilitates the breakdown of vacuolar reserves by hydrolases. It has been proposed that the activation of H^+ -PPase occurs in response to signal transduction intermediates, such as Ca^{2+} and Mg^{2+} , because the abundance of H^+ -PPase did not change after hormonal treatments [47].

4.4. Isoforms

From *Arabidopsis* [59], barley [60], *Chara* [68], and *Acetabularia* [67], only one cDNA for H^+ -PPase has been identified. In mung bean, two cDNAs have been cloned for H^+ -PPase, but the nucleotide sequences of their protein-coding regions were identical to each other [64]. Only a single spot of H^+ -PPase has been detected on an immunoblot of the two-dimensional PAGE of mung bean vacuolar membranes with antibody to a common catalytic sequence of the enzyme [69]. Thus there may not be any isoform of H^+ -PPase in mung bean, *Arabidopsis*, barley, *Chara* or *Acetabularia*.

On the other hand, several cDNAs for H^+ -PPases have been cloned from some plant species, such as red beet (two isoforms) [61], tobacco (at least three isoforms) [62], and rice (two isoforms) [63]. In these cases, the nucleotide sequence of the different clones is highly homologous within the coding region but differs strongly in the untranslated regions. Therefore, these different genes for H^+ -PPase may be differentially, individually regulated in plants. Kim et

al. [61] reported a difference in the transcript levels in leaves and roots of red beet between the two isoforms BVP1 and BVP2. Lerchl et al. [62] isolated 24 cDNA clones for H⁺-PPase from tobacco, and grouped them into three different classes. They found that the levels of mRNAs for these H⁺-PPase isoforms were different in several tissues, such as leaf, stem, root, sepal, and petal. Also, two genes (OVP1, OVP2) of rice H⁺-PPase have been reported to be different in the transcript accumulation in the calli and seedlings [63].

In general, enzyme isoforms are different from each other in enzymatic function, regulatory mechanism of enzyme function, tissue- (or cell-) specificity of gene expression, or growth stage-specificity. There is no report on the difference in the enzymatic activity of H⁺-PPase isoforms, although the organ-specific expression of H⁺-PPase has been reported in several plant species described above. Although a marked difference in the mRNA accumulation was observed in most cases, we cannot discuss the rigidity of gene regulation of H⁺-PPase isoform at the present. The cell specificity in transcription and accumulation of H⁺-PPase protein of each isoform remains to be determined. Generally, H⁺-PPase is thought to be an essential proton pump of vacuolar membranes in any plants as described later. Thus, plant cells may transcribe and translate the H⁺-PPase gene(s) in any tissues at any stages even if their rates may be variable. In some tissues, such as growing tobacco leaves [62], some isoforms may be expressed simultaneously to produce a large amount of H⁺-PPase protein. The content of H⁺-PPase may be regulated in accordance with the demand for the acidification of vacuoles and the active transport of ions and metabolites.

5. Distribution of H⁺-PPase in organisms

5.1. H⁺-PPase in *Trypanosoma* and *Plasmodium*

It is well known that a few photosynthetic bacteria such as *R. rubrum* have H⁺-PPase (PPi synthase) in membranes of their chromatophores [6,7,66]. Vacuolar-type H⁺-PPase has not been found in chloroplasts of plants, although its presence has been expected. No homologues for plant H⁺-PPase have

been detected in the complete genome sequence of a unicellular cyanobacterium (*Synechocystis* sp. PCC6803) or yeast (*S. cerevisiae*). Recently, Scott et al. [93] demonstrated the presence of H⁺-PPase in acidocalcisomes of *Trypanosoma cruzi*. A single-cell eukaryote, *Trypanosoma* is a species of parasitic flagellate protozoans, and the acidocalcisome is a non-lysosomal acidic organelle that is involved in calcium storage within the cell [94]. Relatively high activities (0.09–0.21 $\mu\text{mol/min}$ per mg of membrane protein) have been detected, and the activity was stimulated by K⁺ and was sensitive to Na⁺, and pyrophosphate analogs such as aminomethylene diphosphonate [93]. The primary sequence has been estimated to be similar to plant H⁺-PPase from the immunoreactivity of a protein of 64 kDa with the antibody to plant H⁺-PPase. H⁺-PPase has been thought to drive a Ca²⁺/H⁺ antiporter in acidocalcisomes, although the antiporter has not yet been identified.

Recent progress in the genome projects for many organisms has provided us with a great quantity of information concerning the complete nucleotide sequences and information about the assigned protein-coding genes. A homologue to H⁺-PPase gene has been detected in *Plasmodium falciparum* (database accession no. AF115766). *P. falciparum* is a malaria parasite that belongs to Alveolata. The deduced polypeptide (717 amino acids) contains a sequence DVGADLSGKNE corresponding to a common catalytic site motif. The biochemical properties of this protein remain to be characterized.

5.2. H⁺-PPase in *Chlorophyta*

In addition to *Acetabularia* [25,67] and *Chara* [68,95], both H⁺-PPase and V-ATPase have been demonstrated to exist in the membranes of lytic and contractile vacuoles of another species of Chlorophyta *Chlamydomonas reinhardtii* [96]. The contractile vacuole is a small acidic organelle that functions to expel surplus water from the cell. This organelle possesses the K⁺-stimulated H⁺-PPase activity (0.17 $\mu\text{mol/min}$ per mg) and a protein of 73 kDa immunoreactive with the antibody to H⁺-PPase. *Chlorococcum littorale* also contains both H⁺-PPase and V-ATPase in vacuolar membranes (T. Sasaki, personal communication on a related reference [97]).

The information taken from cDNA cloning revealed that H⁺-PPase of *Chara* rather than *Acetabularia* or *R. rubrum* is more closely related to the enzyme of land plants, suggesting the recent proposal that *Chara* is one of the closest green algae to the land plants [98]. We expect that the evolution of the vacuole in plant cells will be discussed in relation to the molecular evolution of H⁺-PPase, since this enzyme is a characteristic enzyme of plant vacuoles.

5.3. Bacterial H⁺-PPase

Drozdowicz et al. [99] have cloned a DNA for H⁺-PPase of the hyperthermophilic archaebacterium, *Pyrobaculum aerophilum*. This is the first report on bacterial H⁺-PPase except for *R. rubrum* PPi synthase. The DNA encoded a 721-amino-acid polypeptide, and exhibited PPi hydrolysis activity that was not stimulated by K⁺ when expressed in *S. cerevisiae*. Although the physiological function of H⁺-PPase in *P. aerophilum* is unclear, these findings remind us of the existence of the vacuolar-type H⁺-ATPase in archaebacteria. There is a possibility of the more widespread distribution of H⁺-PPase in microorganisms. We will be able to obtain the DNA information about the bacterial H⁺-PPases from the genome projects on various microorganisms in the near future.

5.4. H⁺-PPase in plant plasma membrane

The specific localization of H⁺-PPase in the vacuolar membrane has been demonstrated for many plant species (for review see [1]). It is evident in most cases, but there are a few exceptions. The H⁺-PPase activity has been reported to be associated with the plasma membrane of germinating cotyledons of castor bean [100], and has been observed in the phloem sieve elements in cotyledons and roots of castor bean seedlings by immunochemical techniques [101]. Robinson et al. [102] have also demonstrated the presence of H⁺-PPase in the plasma membrane of developing pea cotyledon. They detected the PPase activity and an immunoreactive protein of 73 kDa, although the H⁺-PPase activity in the plasma membrane was one quarter of that present in the membrane of vegetative vacuole prepared from young cotyledons.

Furthermore, H⁺-PPase has been detected in the plasma membrane of *Chlamydomonas* [96]. The H⁺-PPase activity and the immunoreactive protein with antibody to H⁺-PPase have been detected in the plasma membrane fraction. In plants and other organisms, it has not been examined whether the organelle-specific isoforms of H⁺-PPase are located in the vacuolar and plasma membranes, respectively. The physiological significance of H⁺-PPase localized in plasma membranes is also unclear at present. There is a possibility at least in *Chlamydomonas* that the H⁺-PPase localized in the plasma membrane as a result of the membrane fusion of contractile vacuole with the plasma membrane [96].

6. Further perspectives

It has become clear that the plant vacuolar H⁺-PPase is the most attractive enzyme from the three points of view; namely, the structure–function relationship as a model of proton pump, the molecular evolution as a marker enzyme of plant vacuoles, and the relation to physiological function of plant vacuoles. An elaborate mutational analysis has been done to clarify the functional domains and catalytic residues of H⁺-PPase of plant H⁺-PPases [39–41]. The position of each functional residue should be mapped on the tertiary structure of H⁺-PPase. Our major project on H⁺-PPase is to make two-dimensional crystals in lipid bilayers and/or three-dimensional crystals of this enzyme. A well-known example, the crystallographic analysis of mitochondrial F₁-ATPase, has provided us reasonable information not only on the three-dimensional structure but also on the physical mechanism of enzyme function [103,104].

H⁺-PPase is located in the vacuolar membrane, although there are a few exceptions described above. There is no available information concerning the signal sequence(s) of various kinds of membrane proteins. Nowadays, it is possible to add a tag, such as a His tail and a green fluorescent protein, to the proteins of interest. These analyses will provide us with accurate information about the destination of each H⁺-PPase molecule in plant cells.

All plant vacuoles prepared from various species examined have been demonstrated to contain H⁺-

PPase. Thus, this enzyme is presumably an essential element of giant vacuoles in plant cells. During evolution of organisms, ancestral plant species obtained H^+ -PPase in addition to vacuolar-type H^+ -ATPase. Probably, acquisition of H^+ -PPase enables vacuoles and plant cells to expand. The molecular evolution of H^+ -PPase remains to be elucidated. The level of H^+ -PPase changes according to the physiological conditions and in response to environmental stresses. The regulatory mechanism of H^+ -PPase gene expression and the posttranslational regulation remain to be resolved. Plant vacuolar H^+ -PPase is one of the rare, plant-origin enzymes that can provide useful information to general molecular biology and bioenergetics.

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