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VACUOLAR-TYPE H^+-TRANSLOCATING ATPases IN PLANT ENDOMEMBRANES: SUBUNIT ORGANIZATION AND MULTIGENE FAMILIES

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Summary

Acidification of endomembrane compartments by the vacuolar-type H^+-translocating ATPase (V-ATPase) is vital to the growth and development of plants. The V-ATPase purified from oat roots is a large complex of $650\times10^3\,M_r$ that contains 10 different subunits of 70, 60, 44, 42, 36, 32, 29, 16, 13 and $12\times10^3\,M_r$. This set of ten polypeptides is sufficient to couple ATP hydrolysis to proton pumping after reconstitution of the ATPase into liposomes. Unlike some animal V-ATPases, the purified and reconstituted V-ATPase from oat is directly stimulated by Cl^-. The peripheral complex of the ATPase includes the nucleotide-binding subunits of 70 and $60\times10^3\,M_r$ and polypeptides of 44, 42, 36 and $29\times10^3\,M_r$. Six copies of the $16\times10^3\,M_r$ proteolipid together with three other polypeptides are thought to make up the integral sector that forms the H^+-conducting pathway. Release of the peripheral complex from the native membrane completely inactivates the pump; however, the peripheral subunits can be reassembled with the membrane sector to form a functional H^+ pump.

Comparison of V-ATPases from several plants indicates considerable variations in subunit composition. Hence, several forms of the V-ATPase may exist among, and probably within, plant species. At least four distinct cDNAs encode the $16\times10^3\,M_r$ proteolipid subunit in oat. Multiple genes could encode different subtypes of the H^+ pump that are regulated by the developmental stage and physiological function specific to the cell or tissue type.

Introduction

In plants, several different electrogenic H^+ pumps provide the energy required to take up and distribute essential mineral nutrients for growth and development. These primary active transporters are (i) a plasma membrane H^+-ATPase, (ii) a vacuolar-type H^+-ATPase and (iii) an H^+-pumping pyrophosphatase (H^+-PPase). The electrochemical gradient generated by these H^+ pumps provides the driving force for the secondary transport of numerous ions and metabolites (Fig. 1) (Sze, 1985).

Two distinct H^+ pumps, the V-ATPase and the H^+-PPase, acidify the vacuolar compartment (Rea and Sanders, 1987). Meristematic plant cells contain numerous small vacuoles or provacuoles that originate from the *trans* Golgi network. As cells

Key words: H^+-ATPase, proton pump, vacuolar membrane, V-ATPase, plant, transport.

differentiate and elongate, the provacuoles fuse to form one or more large vacuoles characteristic of plant cells. In mature cells, the vacuole is the largest intracellular organelle, occupying about 90% of the cell volume, surrounded by a membrane called the tonoplast. Being dynamic organelles, vacuoles participate in diverse functions (Table 1) depending on the tissue, the stage of development and the signals received. These functions include transport and storage of ions and metabolites, osmoregulation, signal transduction, protein storage and turnover, and storage of secondary metabolites and pigments (see Sze *et al.* 1992; Boller and Wiemken, 1986). Fig. 1 shows some of the ion channels and H^+-coupled transporters of plant vacuoles that are dependent on the primary H^+ pumps. The proton-motive force, generated by either the H^+-ATPase or the H^+-PPase, and the resulting ion and metabolite fluxes are essential or central to the vital cellular processes performed by vacuoles and other endomembranes.

V-ATPases may be an integral component of the endomembrane system in plants (Sze *et al.* 1992), as has been observed in animals (Forgac, 1989). Plant endomembranes, which include the Golgi network, clathrin-coated vesicles, secretory vesicles and plasma membrane as well as the tonoplast, play a major role in the biogenesis of organelles, in the deposition of materials within the organelle and in the biosynthesis and transport of

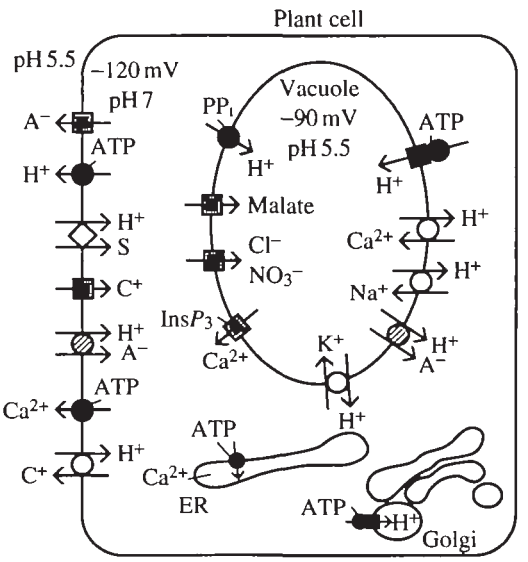


Fig. 1. Model of primary H^+ pumps, H^+-coupled transporters and channels in a simplified plant cell. A plasma membrane ATPase (P-type) pumps H^+ out of the cell, generating a proton electrochemical gradient (inside −120 mV relative to the outside). An electrogenic V-ATPase and an H^+-PPase (pyrophosphatase) acidify the vacuole. The proton-motive force provides energy for uptake and release of solutes across the tonoplast through antiporters (open circles), symporters (hatched circles) and channels (squares). Primary ion pumps are shown as filled circles. C^+, A^- and S refer to cations, anions and organic solutes, respectively. The V-ATPase also acidifies endomembrane compartments, such as the Golgi body and coated vesicles. $Ins P_3$, inositol triphosphate; ER, endoplasmic reticulum.

material destined for extracellular secretion (Chrispeels, 1991). One important feature of the secretory pathway is the role of organelle acidification in transport and targeting, as in the Golgi compartments (Mellman *et al.* 1986). Evidence for acidification of the Golgi compartments and coated vesicles by a vacuolar-type H^+-ATPase in plants is emerging (e.g. Chanson and Taiz, 1985; Depta *et al.* 1991).

In the last 10 years, remarkable progress has been made in understanding the structure and function of V-ATPases from plants (Sze *et al.* 1992), fungi (Bowman and Bowman, 1986) and animals (Forgac, 1989; Nelson and Taiz, 1989). The unique characteristics of the H^+ pumping (pH gradient, ΔpH and membrane potential, $\Delta\varphi$) and ATP hydrolysis activities in vacuolar membrane vesicles from plants have been reviewed by Sze (1985). The central role of the V-ATPase in the growth and development of plants has been discussed (Sze *et al.* 1992). This chapter will highlight recent advances concerning the complex structure of the plant V-ATPase using data mainly from oat roots. Comparisons with V-ATPases from other plants and from animal tissues are discussed briefly. For a comprehensive coverage of plant V-ATPases, several other reviews are available (Sze, 1985; Sze *et al.* 1992; Rea and Sanders, 1987).

Table 1. *Key functions performed by plant endomembranes*

Organelle	Function
Vacuoles	Generate and maintain cell turgor pressure
	e.g. during cell expansion and elongation
	opening/closing of stomatal guard cells
	Transport and store ions and metabolites
	e.g. K^+, Cl^-, Ca^{2+}
	malate (especially CAM plants),
	sucrose (sugar cane)
	Regulate and maintain suitable cytoplasmic conditions
	e.g. Ca^{2+}, pH
	Store proteins, secondary metabolites, pigments
	e.g. zein (corn seeds),
	nicotine (tobacco leaves),
	anthocyanins (tulip petals)
	Contain many acidic hydrolases and defense proteins
	e.g. phosphatases, proteases, lipases
	to degrade macromolecules,
	chitinase, legume lectins
Coated vesicles	Transport proteins from Golgi complex to storage vacuoles
	e.g. lectin precursor in developing pea cotyledons
Golgi complex	Process and sort membrane and organellar proteins
	Synthesize and secrete cell wall matrix polysaccharides
	e.g. pectins and hemicellulose
	Process and secrete glycoproteins
	e.g. extensin

From Taiz and Zeiger (1991), Boller and Wiemken (1986), Robinson and Depta (1988) and Chrispeels (1991).

A functional H^+-pumping ATPase is a large complex of 10 subunits

Initial purification of plant V-ATPases had revealed three major subunits (Rea and Sanders, 1987); however, recent studies show that the plant enzyme is as complex as those found in animal and yeast endomembranes. The V-ATPase from oat roots was solubilized with Triton X-100, and purified by gel filtration and ion exchange chromatography (Ward and Sze, 1992*a*). Nitrate-sensitive ATP hydrolysis is associated with a large complex of 650×10^3 M_r which contains subunits of 70, 60, 44, 42, 36, 32, 29, 16, 13 and 12×10^3 M_r. In general, the subunit compositions of the V-ATPases purified from red beet, mung bean, oat and barley are similar, though not identical (Table 2). Until recently, none of the purified plant V-ATPases had been tested for proton transport activity in a reconstituted system.

To determine whether the V-ATPase complex from oat was active in proton transport, the purified enzyme was incorporated into liposomes from *Escherichia coli* phospholipids by removing the Triton X-100 with SM-2 biobeads. Acidification of K^+-loaded proteoliposomes, monitored by the quenching of Acridine Orange fluorescence, was stimulated by valinomycin (Fig. 2A). As the presence of K^+ and valinomycin dissipates a transmembrane electrical potential, the results indicated that ATP-dependent H^+ pumping is electrogenic. The reconstituted pump retained its native properties since bafilomycin, a specific inhibitor of V-ATPases, completely inhibited H^+ pumping at concentrations less than $50\,nmol\,l^{-1}$ (Fig. 2B). Thus, a set of ten polypeptides associated with the oat V-ATPase (Table 2) is sufficient to couple ATP hydrolysis to proton pumping.

Table 2. *Subunit composition and function of V-ATPases from plant tissues and selected animal sources*

Source	$M_r\times10^{-3}$	Subunit composition ($\times10^{-3}$ M_r)											References
Plant													
Red beet		100	**67**	**55**†	**52**	**44**				**32**	16‡		1
Mung bean			68	57		44	38	37	32		16	13 12	2
Barley root		115	68	53		45	42	34	32		17	13 12	3
Oat root	650		**70***	**60**		**44**	**42**	**36**	32	**29**	16‡	13 12	4
Animal													
Liver or kidney Golgi body			**72**	**57**		**41**		**34**	**33**		16		6
Kidney plasma membrane	586		70	56		45	42	38	33	31	15	14 12	7
Bovine coated vesicle	760	100_1	$\mathbf{73}_3$	$\mathbf{58}_3$		$\mathbf{40}_1$	38_1	$\mathbf{34}_1$	$\mathbf{33}_1$	19_1	17_6		5

*70×10^3 M_r binds Nbd-Cl catalytic subunit.

†55×10^3 M_r binds Bz-ATP regulatory subunit.

‡16×10^3 M_r binds DCCD proteolipids form a channel for H^+ translocation.

(1) Parry *et al.* (1989); Manolson *et al.* (1985); Rea *et al.* (1987*a*). (2) Matsuura-Endo *et al.* (1990). (3) DuPont and Morrissey (1992). (4) Randall and Sze (1987); Kaestner *et al.* (1988); Ward and Sze (1992*a*,*b*). (5) Forgac (1989). (6) Moriyama and Nelson (1989); Young *et al.* (1988). (7) Gluck and Caldwell (1987); Wang and Gluck (1990).

Subunits in bold print have been shown to be peripheral.

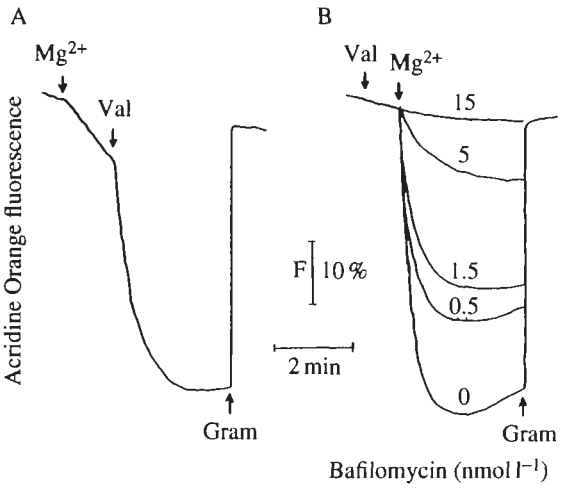


Fig. 2. (A,B) Purified and reconstituted V-ATPase from oat roots is a functional H^+ pump. Proteoliposomes (approximately 2 μg of protein) were diluted into a mixture containing K_2SO_4, Hepes–BTP pH 7, 1.5 mmol l^{-1} ATP and 0.5 μmol l^{-1} Acridine Orange. H^+ pumping was started by adding $MgSO_4$ (final concentration 5 mmol l^{-1}). When present, bafilomycin in dimethylsulfoxide (DMSO) was added to the incubation mixture at 22 °C for 10 min prior to the assay. Final concentration of valinomycin (Val) was 0.5 μmol l^{-1} and of gramicidin (Gram) was 5 μg ml^{-1} (from Ward and Sze, 1992*b*). In B, varying concentrations of bafilomycin up to 15 nmol l^{-1} were added. F, fluorescence intensity.

Unlike the purified red beet or barley H^+ pump, the oat V-ATPase does not contain a large subunit of 100×10^3–115×10^3 M_r. In the bovine coated vesicle V-ATPase, a 100×10^3 M_r subunit is required for H^+ transport, though it is particularly sensitive to proteases (Adachi *et al.* 1990). Since the purified oat V-ATPase lacks such a subunit and is active in transport, we conclude that the oat V-ATPase enriched in roots of 4-day-old seedlings lacks a 100×10^3 M_r subunit. The variations in subunit composition (Table 2) would suggest the presence of several subtypes of V-ATPases among plant species and probably within them as well. Interestingly, the oat root V-ATPase is strikingly similar to the kidney microsomal or brush border V-ATPase in its subunit composition and in its enzymatic properties (Gluck and Caldwell, 1987; Wang and Gluck, 1990). Furthermore, like the oat V-ATPase, the Golgi-associated V-ATPase from rat liver lacks a 115×10^3 M_r subunit (Moriyama and Nelson, 1989) (Table 2). Differences in subunit composition between ATPases of various plants (Table 2) suggest that the 100×10^3 M_r, 52×10^3 M_r and 36×10^3 M_r subunits are either compartment- or tissue-specific or are developmentally regulated.

ATP hydrolysis of the purified and reconstituted ATPase is tightly coupled to H^+ translocation. Both ATP hydrolysis and H^+ transport activities were 50 % inhibited by bafilomycin at 1 nmol per mg protein. With a relative molecular mass of 650×10^3 for the V-ATPase, this inhibition would indicate there are 0.65 nmol of bafilomycin per nmol of ATPase. Hence, bafilomycin reacts stoichiometrically with the V-ATPase at a site that has yet to be determined.

Unlike some of the animal V-ATPases, the oat V-ATPase is directly stimulated by Cl^- and this activation is coupled to H^+-pumping. Proton transport by the bovine coated vesicles is dependent on permeant anions that lead to dissipation of the membrane potential; however, neither ATP hydrolysis nor H^+ pumping by the purified and reconstituted coated-vesicle H^+-ATPase is stimulated by Cl^- (Arai *et al.* 1989). By contrast, Cl^- stimulates the purified and reconstituted oat V-ATPase and the activation is coupled to H^+ pumping (Ward and Sze, 1992*b*). Since cytoplasmic [Cl^-] in plant cells is about 30–90 mmol l^{-1}, direct Cl^- stimulation of the V-ATPase may be important for regulating the pH gradient (ΔpH) and membrane potential ($\Delta\varphi$) across the vacuolar as well as the Golgi compartments.

The large peripheral complex can be dissociated from the membrane integral sector and then reassembled

The nucleotide-binding subunits of 70 and 60×10^3 M_r were previously shown to be peripheral subunits, as they are released from the membrane by chaotropic ions (Rea *et al.* 1987*a*), low ionic strength solutions or 0.1 mmol l^{-1} EDTA (Lai *et al.* 1988). However, recent studies clearly show that the peripheral complex of the oat V-ATPase consists of six different polypeptides of 70, 60, 44, 42, 36 and 29×10^3 M_r (Fig. 3A). Like V-ATPases from chromaffin granules or bovine coated vesicles, 0.1 mol l^{-1} KI in the presence of 5 mmol l^{-1} MgATP is more effective in stripping off the peripheral complex than KI alone. Interestingly, near physiological concentrations of KCl (0.2 mol l^{-1}) or NaCl (0.15 mol l^{-1}) also released the peripheral subunits, which inactivated 60–80 % of the H^+-pumping and ATPase activities (Parry *et al.* 1989; Ward *et al.* 1992). In red beet, the peripheral complex consists of five polypeptides of 67, 55, 52, 44 and 32×10^3 M_r.

Five of the peripheral polypeptides from oat V-ATPase are released as a complex of about 540×10^3 M_r from the native membrane. Surprisingly, the 42×10^3 M_r subunit is not associated with this complex, but appears to be released separately (Ward and Sze, 1992*a*). These findings are strikingly similar to the dissociated V-ATPase of bovine coated vesicles, in which a 40×10^3 M_r subunit is not associated with the V_1 subcomplex of 500×10^3 M_r. Recently, Forgac and coworkers (Puopolo *et al.* 1992) showed that the 40×10^3 M_r subunit is not required for H^+ pumping activity. Since V_1V_o complexes are less stable when assembled in the absence than in the presence of a 40 or 34×10^3 M_r subunit, they suggest that these subunits stabilize the ATPase complex. Functionally, it is possible that the 42×10^3 M_r subunit from the oat V-ATPase is analogous to the 40×10^3 M_r subunit of the coated-vesicle proton pump.

Unlike the F_o complex of F-type ATPases, the membrane integral sector of the V-ATPase, the V_o complex, does not form a leaky proton pore. Although KI and MgATP completely abolished ATP-dependent proton pumping in tonoplast vesicles, PP_i-driven H^+ pumping was not affected (Rea *et al.* 1987*a*; Ward and Sze, 1992*a*). The H^+-PPase, localized on the same membrane as the V-ATPase in plants, is made up of 2–4 integral polypeptides of 81×10^3 M_r that contain at least 13 trans-membrane domains (Sarafian *et al.* 1992). The ability to maintain a pH gradient in V_1-stripped vesicles suggests that the V_o complex does not conduct H^+ passively.

The subunit composition of the V_o complex, which forms the proton-conducting pathway, is less understood in both plants and animals than is that of the V_1 complex. The major component common to the V_o complex of all V-ATPases is the *N,N'*-dicyclohexylcarbodiimide (DCCD)-binding $16\times10^3 M_r$ subunit (Forgac, 1989; Nelson and Taiz, 1989). This hydrophobic polypeptide is referred to as a proteolipid because of its solubility in chloroform/methanol. From DCCD-binding studies and partial purification of the proteolipid from oat vacuolar vesicles, we estimated that there are six copies of the $16\times10^3 M_r$ subunit per V_o complex (Kaestner *et al.* 1988). Based on elimination of the peripheral polypeptides, we have suggested that the $16\times10^3 M_r$ subunit together with possibly the 32, 13 and $12\times10^3 M_r$ subunits make up the V_o complex from oat roots (Fig. 3A). However, in red beet V-ATPase, the V_o sector would consist of the $16\times10^3 M_r$ proteolipids plus the $100\times10^3 M_r$ polypeptide (Parry *et al.* 1989).

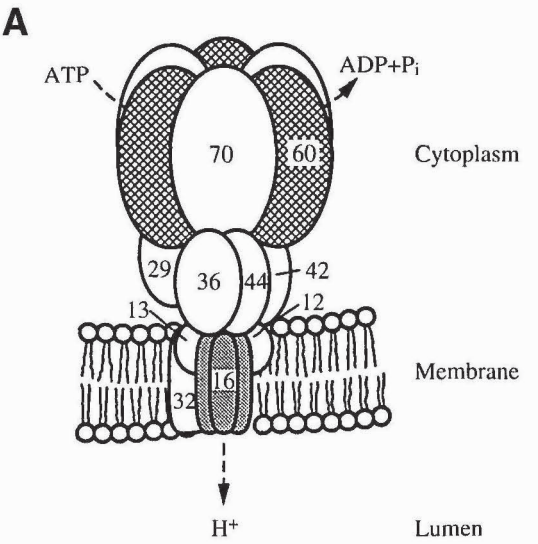
Dissociation and inactivation of the oat V-ATPase induced by KI is reversible *in vitro* (Ward *et al.* 1992) and possibly *in vivo*. As for the bovine-coated vesicle H^+-ATPase (Puopolo and Forgac, 1990), removal of KI and MgATP by dialysis restores activity to the oat V-ATPase. ATP hydrolysis activity increased to about 50% of that of the untreated control, and the ATPase activity was coupled to H^+ pumping as seen from the recovery of H^+ transport. Furthermore, disappearance of the solubilized 70 and $60\times10^3 M_r$ subunits from the supernatant confirmed that the V_1V_o complex had reassembled during dialysis. It is unclear whether such reversible dissociation of the V-ATPase in animal cells holds any physiological significance. In plants, many multimeric enzymes dissociate in response to chilling. The loss of vacuolar H^+-pumping ATPase activity in mung bean suspension cells incubated at 2 °C (Yoshida, 1991) is probably caused by enzyme dissociation, which is enhanced at 4 °C (Parry *et al.* 1989; Ward *et al.* 1992). Full activity is restored within 1 h after cells are returned to 22 °C. Since recovery did not require *de novo* protein synthesis (Yoshida, 1991), the dissociated and inactive V-ATPase is suggested to have reassembled at 22 °C. Hence, the ability to dissociate and reassociate may be one mechanism by which the V-ATPase is regulated *in vivo* in response to environmental stress.

Abundance and transport rate of V-ATPase in plant cells

Electron microscopy demonstrates that the plant V-ATPase is a major component of the vacuolar membrane in actively growing tissues. The peripheral sectors of the plant V-ATPases are visible on the surface of vacuolar vesicles as knob-like structures (Fig. 3B). Negatively stained vesicles reveal densely packed particles 10–12 nm in diameter. These particles are removed from the surface after washing with KI or KNO_3, supporting their identity as the V_1 complex of the V-ATPase (Ward *et al.* 1992). Close examination of the surface view shows a patchy distribution of structures. Some of the particles appear trigonal whereas others contain six subparticles (Taiz and Taiz, 1991; Klink and Luttge, 1991).

The abundance of the V-ATPase enzyme is supported by purification studies. A purification of 6- to 12-fold is sufficient to obtain the purified enzyme from oat roots, mung bean hypocotyl or barley roots of young seedlings (Ward and Sze, 1992*a*;

Matsuura-Endo *et al.* 1990; DuPont and Morrisey, 1992). This value indicates that the V-ATPase makes up about 8–16 % of the vacuolar membrane protein in young, actively growing seedlings. By contrast, V-ATPase appears to be only 1–2 % of the tonoplast protein in mature red beet roots (Parry *et al.* 1989). The relative abundance of V-ATPases in young tissues probably reflects the presence of more H^+ pumps in Golgi membranes, which are active in sorting and secretion of cell wall matrix polysaccharides and glycoproteins (Table 1).



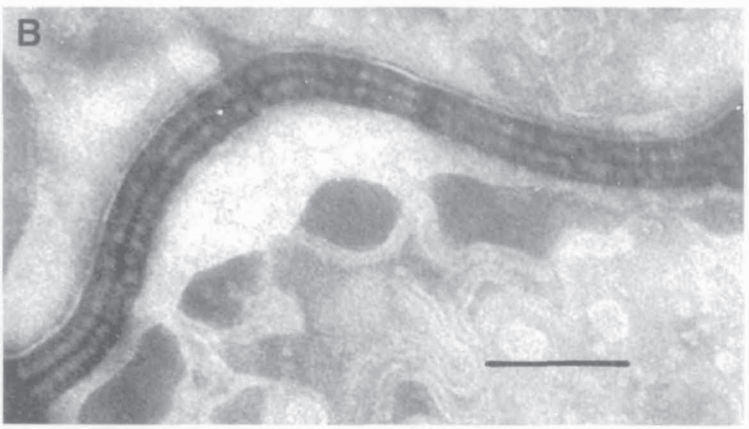


Fig. 3. Structure of a V-ATPase from oat roots. (A) Structural model. A large peripheral complex includes the 70×10^3 M_r (catalytic) and the 60×10^3 M_r (nucleotide-binding) subunits plus several accessory subunits of unknown function. Six copies of the DCCD-binding 16×10^3 M_r proteolipid together with other integral subunits are thought to form a pore for H^+ transport. (B) V-ATPases appear as knob-like structures on tonoplast vesicles from oat roots. Negatively stained vesicles reveal dense patches of knobs (approximately 10–12 nm in diameter). Scale bar, 100 nm.

The turnover number of protons pumped by V-ATPases from plants is within the range of 10^2–10^3 ions s^{-1} typical of ion pumps driven by ATP hydrolysis. With the exception of red beet, the specific activities of purified V-ATPases from plant tissues range from 2.7 μmol ADP mg^{-1} protein min^{-1} at 22 °C (Randall and Sze, 1986; Ward and Sze, 1992*a*) to 4.1 μmol ADP mg^{-1} protein min^{-1} at 30 °C (Matsuura-Endo *et al.* 1990). With a stoichiometry of $2H^+$ pumped per ATP hydrolysed (see Sze *et al.* 1992 and references therein) and an average relative molecular mass of 650×10^3 for the V-ATPase (Ward and Sze, 1992*a*), the transport rates of vacuolar H^+-ATPase from oat (Ward and Sze, 1992*a*) and mung bean (Matsuura-Endo *et al.* 1990) are 60 and 90 protons per second, respectively. These transport rates are comparable to the bovine kidney V-ATPase, which has specific activities as high as 3.1 μmol mg^{-1} protein min^{-1} (Gluck and Caldwell, 1987).

Molecular structure and function of the 16×10^3 M_r proteolipid

The 16×10^3 M_r proteolipid is one of the most interesting subunits of the V-ATPase complex because it is similar to the 8×10^3 M_r proteolipid of the F_o-ATPase and has a putative role in H^+ translocation. DCCD inhibits H^+-pumping and ATP hydrolysis activity by covalent modification of the 16×10^3 M_r subunit from oat or red beet V-ATPase (Kaestner *et al.* 1988; Rea *et al.* 1987*b*). The amount of [^{14}C]DCCD bound to the 16×10^3 M_r polypeptide is directly proportional to the inhibition of ATPase activity. From purification studies, we estimate that there are approximately six copies of the 16×10^3 M_r proteolipid per ATPase complex. However, binding studies and the kinetics of noncooperative inhibition show that the enzyme is completely inactivated when only 1 mol of DCCD is bound per mol of ATPase.

To understand the structure and function of the proteolipid at the molecular level, we have obtained a cDNA encoding the complete 16×10^3 M_r subunit from oat roots. This cDNA was detected using a synthetic oligonucleotide corresponding to a region of the V-ATPase proteolipid from bovine chromaffin granule (Mandel *et al.* 1988). The predicted amino acid sequence (165 amino acids, relative molecular mass 16641) of the oat proteolipid revealed a molecule with four membrane-spanning domains (Fig. 4) (Lai *et al.* 1991) similar to other V-ATPase proteolipids. Unlike the 8×10^3 M_r proteolipid from F-ATPases, the amino acid sequence of the 16×10^3 M_r subunit of V-ATPases is conserved among eukaryotes, especially in transmembrane domain IV. Domain IV shows about 80% amino acid sequence identity between the oat and the yeast 16×10^3 M_r proteolipid. A glutamate residue within this hydrophobic region is thought to be modified by the inhibitor DCCD. Support for this hypothesis has been provided in yeast, where replacing glutamate-137 with several amino acids, except aspartate, abolished activity (Noumi *et al.* 1991). Based on analogies with the 8×10^3 M_r proteolipid, one can envisage a simple model in which a functional proton pore is formed from six copies of the 16×10^3 M_r proteolipid plus a few other polypeptides. DCCD modification of only one of these proteolipids blocks H^+ conductance by modifying the cooperative interaction required among the six subunits to form the proton pathway.

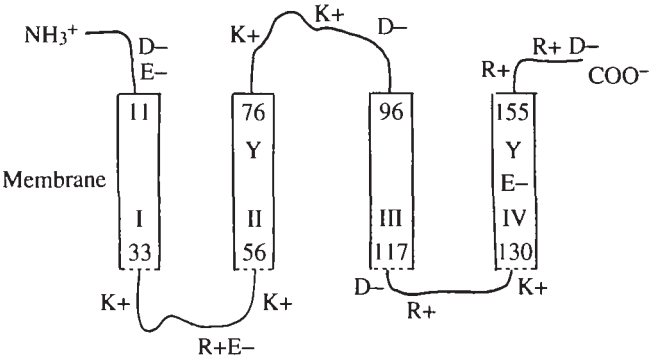


Fig. 4. Structural model of the $16\times10^3 M_r$ proteolipid subunit from the oat V-ATPase. Four membrane-spanning domains (I–IV) are suggested by the hydropathy profile (Lai *et al.* 1991). Numbers within each transmembrane region refer to the amino acid residue number. Single letters indicate the locations of charged amino acids. Although the orientation of the V-ATPase proteolipid within the membrane is not clear, according to the model of the $8\times10^3 M_r$ proteolipid of the F_o-ATPase, both N and C termini face the exoplasmic side (i.e. lumen face).

Biosynthesis and orientation of the $16\times10^3 M_r$ subunit

Although hydropathy plots predicted a molecule with four membrane-spanning domains (Fig. 4), the topology of the molecule within the membrane is not clear. As a first step towards resolving this question, we have examined the orientation of the $16\times10^3 M_r$ subunit synthesized *in vitro*. *In vitro* transcribed RNA was obtained using T_7 RNA polymerase and the cDNA encoding the complete sequence of the oat $16\times10^3 M_r$ proteolipid as a template. The RNA was translated using rabbit reticulocyte lysate and the products labeled with [^{35}S]methionine were analysed by SDS–PAGE and fluorography. A $16\times10^3 M_r$ polypeptide translated *in vitro* was stably inserted into either dog pancreatic microsomes or oat microsomal vesicles (S. Lai and H. Sze, unpublished results). The product was soluble in chloroform/methanol, but resistant to treatment with 0.4 mol l^{-1} Na_2CO_3 at pH 11, which releases peripheral proteins from the membrane.

To test the orientation of the $16\times10^3 M_r$ proteolipid, we made two assumptions: (i) the $16\times10^3 M_r$ proteolipid synthesized *in vitro* inserts into the membrane with the same orientation as the native proteolipid, and (ii) the protein inserts only into cytoplasmic-side-out endoplasmic reticulum vesicles. If so, we can examine the orientation based on the sensitivity of the polypeptide to proteases. Our working hypothesis is that only regions exposed at the membrane surface are sensitive to proteases. If the N and C termini face the cytoplasmic (cyt) side, protease digestion should yield small peptides of about $6–8\times10^3 M_r$. However, should the N and C termini face the lumen (exo) side, proteolysis would result in peptides of about $6–8\times10^3 M_r$ and small fragments of $3–4\times10^3 M_r$.

Our preliminary results would support a model with C_{cyt} and N_{cyt}. After *in vitro* translation with dog pancreatic microsomes, the membranes were pelleted and digested with trypsin or proteinase K in the absence or presence of Triton X-100. In the absence of Triton X-100, either trypsin or proteinase K produced polypeptides of $6–8\times10^3 M_r$ and

$16\times10^3 M_r$. However, polypeptides of $4–5\times10^3 M_r$, but not $8\times10^3 M_r$, were detectable after protease digestion in the presence of Triton X-100.

This assignment in orientation is not consistent with the model of the F-ATPase, in which the $8\times10^3 M_r$ proteolipid has both C_{exo} and N_{exo}. Using peptide-specific antibodies, Hensel *et al.* (1990) showed that the conserved hydrophilic region of subunit *c* is exposed to the cytoplasmic side. Furthermore, the proposed topology of the oat 16×10^3 M_r proteolipid does not agree with the model proposed by Hartmann *et al.* (1989), in which the more positive portion of residues flanking the first internal signal-anchor sequence of the protein faces the cytosol. Clearly, further studies are needed to confirm the proposed orientation of the $16\times10^3 M_r$ proteolipid, in order to understand the biosynthesis and assembly of the V-ATPase complex.

V-ATPase subunits are encoded by multigene families

A gene family of at least four members encodes the $16\times10^3 M_r$ proteolipid in oats (Lai *et al.* 1991). The four distinct cDNAs showed extensive divergence in their codon usage and in their 3′-untranslated regions; however, the deduced amino acid sequences were 97–99 % identical. Genomic Southern blot analysis suggests that there may be as many as 6–7 members in this gene family from oat.

Since oat is a hexaploid, we have begun to study the physiological significance of multiple genes encoding V-ATPase subunits using *Arabidopsis*. *Arabidopsis thaliana* has a small haploid genome (70 000 kb), and tends to have smaller gene families. Using the cDNA from oat as a probe, at least two similar, but distinct, cDNAs have been obtained. These two differed from each other mainly in their codon usage and in their 3′-untranslated regions. The primary amino acid sequences deduced from the two clones, AVA-P1 and AVA-P2 (*Arabidopsis* *V*acuolar *A*TPase-*p*roteolipid), were identical. Genomic Southern analyses showed that 3–4 DNA fragments hybridized with AVA-P1 at high stringency. These results suggest that *Arabidopsis* also has a small gene family encoding the $16\times10^3 M_r$ subunit (I. Perera and H. Sze, unpublished results).

The $70\times10^3 M_r$ subunit may also be encoded by a multigene family in several plants. Partial sequences (252–294 bp in length) obtained by amplifying genomic DNA with conserved primers indicate that at least two separate genes may encode the catalytic subunit in *Psilotum* and *Equisetum*, two early land plants (Starke *et al.* 1991). The two DNA fragments of *Psilotum* differ mainly in their codon usage. In carrots, preliminary results using a similar approach suggest there are at least three separate genes for the $70\times10^3 M_r$ subunit (L. Taiz, personal communication). However, it is not clear whether these multiple genes are actually expressed.

The discovery of multiple genes encoding V-ATPase subunits in plants raises many interesting questions. Perhaps multiple genes encode isoforms of the enzyme which could be differentially localized or specifically regulated, depending on the developmental stage. The participation of V-ATPases in the diverse roles of plant endomembranes would necessitate that the activity and expression of these pumps should be under physiological and developmental regulation. One future challenge in this area will be to

understand how H^+ pumping by V-ATPases and solute fluxes are integrated into plant cell division, growth and development.

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