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MOLECULAR CHARACTERIZATION OF A PYROPHOSPHATE-ENERGIZED PROTON PUMP

Vahé Sarafian

Biology Department
McGill University, Montréal
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A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy

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To Diane and Isabelle,

ABSTRACT

The H^+-translocating inorganic pyrophosphatase from vacuolar membranes of red beet storage roots (Beta vulgaris L.) was purified after solubilization in Triton X-100 through a combination of anion-exchange and size exclusion chromatographies. SDS-PAGE showed strong correlation between a 67 kDa polypeptide and pyrophosphatase activity. Radiation-inactivation studies of the H^+-PPase indicate a functional size of 91 kDa for hydrolysis and 320 kDa for H^+ translocation, suggesting an oligomeric structure for the holoenzyme. Affinity purified antibodies were used to screen cDNA libraries of Arabidopsis thaliana yielding clones which contained sequences matching amino acid sequences obtained from tryptic fragments of the 67 kDa hydrolytic subunit. The predicted protein is highly hydrophobic with a molecular size of 81 kDa. Southern analyses show a single copy for the H^+-PPase in Arabidopsis. The lack of sequence identities between the H^+-PPase and other known proteins implies a novel class of ion translocases.

RESUME

La H^+-pyrophosphatase inorganique des membranes vacuolaires a été purifiée des racines de la betterave rouge (Beta vulgaris L.) après solubilisation avec du Triton X-100. Des chromatographies d'échange ionique et de filtration sur gel ont produit, par identification sur gel acrylamide SDS, une polypeptide de 67 kDa qui démontrait une forte corrélation avec l'activité de la pyrophosphatase. L'analyse par la méthode d'inactivation par irradiation, a révélé des masses moléculaires fonctionnelles de 91 kDa pour la fonction hydrolytique et de 320 kDa pour la fonction de transport. Une structure oligomérique est proposée pour l'enzyme. Une banque ADNc d'Arabidopsis thaliana a été criblée avec des anticorps purifiés par affinité. Les clones obtenus contenaient des séquences similaires à des séquences d'acides aminés obtenues par digestion tryptique de la sous-unité hydrolytique de 67 kDa. La protéine codée par les clones est extrêmement hydrophobique et son poids moléculaire est de 81 kDa. La séquence de la H^+-PPase ne ressemble à aucune autre protéine connue, ce qui suggère qu'elle appartient à une nouvelle catégorie de translocases d'ions.

TABLE OF CONTENTS

CHAPTER 2. **PURIFICATION OF AN H^+-TRANSLOCATING INORGANIC PYROPHOSPHATASE FROM VACUOLE MEMBRANES OF RED BEET**

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PREFACE

As required by the Faculty of Graduate Studies and Research of McGill University the following statement is quoted:

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This thesis consists of an Abstract, Résumé, Introduction, Literature Review (Chapter 1), Results (Chapters 2, 3 and 4) presented in manuscript form, General Discussion (Chapter 5) and Literature Cited.

Chapter 2 has been published in **Plant Physiology** as:

Sarafian, V. and Poole, R.J. 1989. Purification of an H^+-Translocating Inorganic Pyrophosphatase from Vacuole Membranes of Red Beet. **Plant Physiol.** 91:34-38

Chapter 3 was published in the **Biochemical Journal** as:

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Chapter 4 has been published in the **Proceedings of the National Academy of Sciences, USA** as:

Sarafian, V., Kim, Y., Poole, R.J. and Rea, P.A. 1992. Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump (H^+-PPase) of Arabidopsis thaliana. **Proc. Natl. Acad. Sci. U.S.A.** 89:1775-1779

To ensure homogeneity among the chapters, some minor editing of the manuscripts has been made. Chapter 4 was accepted for publication in its present form but the published version was reduced because of the page limit policy of the Journal.

The literature cited in this thesis has been combined and placed at the end of the dissertation.

All the results cited are solely the work of the candidate with the exception of:

- Figure 4 in chapter 4. Northern blot analysis of Arabidopsis tonoplast H^+-PPase. Preparation of RNA and the Northern blot were done by Dr. Y. Kim

- Figure 5 in chapter 4. Genomic Southern analysis of tonoplast H^+-PPase. Preparation of genomic DNA and the Southern blot were performed by Dr. Y. Kim.

Materials and services obtained from elsewhere, e.g. antibodies, cDNA libraries, peptide sequencing, etc., are explicitly mentioned in the appropriate Materials and Methods sections.

ABBREVIATIONS

BSA	bovine serum albumin
BTP	1,3-bis[tris(hydroxymethyl)methylamino]-propane
CHAPS	3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate
D_{37}	radiation dose required to decrease enzyme activity to 37% of initial rate
DTT	dithiothreitol
EB	20 mM His-Cl(pH 6.0)/10% glycerol/0.25% Triton X-100/40 mM KCl/2 mM $MgCl_2$/5 mM DTT
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis(ß-aminoethyl ether)
Mes	2-(N-morpholino) ethanesulfonic acid
NEM	N-ethylmaleimide
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
PPi	inorganic pyrophosphate (diphosphate)
PVP	polyvinylpyrrolidone (insoluble)
PVP-40	polyvinylpyrrolidone (average mol. wt. 40,000)
RB	30 mM BTP-Mes(pH 8.0)/10% glycerol/1% CHAPS/25 mM KCl/4mM $MgCl_2$/1mM BTP-EDTA/10mM DTT/0.03% NaN_3/ 2.0 mg/ml phospholipid
S.E.M.	standard error of the mean
SDS	sodium dodecyl sulfate
SM	5 mM BTP-Mes(pH 8.0)/10% glycerol/1 mM BTP-EDTA/ 5 mM DTT
Tris	tris(hydroxymethyl)aminomethane
TSB	Tris Saline Buffer (10 mM Tris-Cl pH 7.3/0.9% NaCl)
TTSB	0.05% Tween 20 in TSB

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

During the past two decades our understanding of vacuolar function in plants has greatly improved. From an earlier concept of the vacuole as a static storage compartment for unwanted chemicals, the current perception is that of a dynamic organelle, whose contents are continuously exchanged with the cytoplasm. In their review of vacuolar compartmentation, Boller & Wiemken (1986) compare the homeostatic role of the vacuole with that of body fluids in animals. Indeed, the continuous interplay between the vacuolar and cytoplasmic compartments seems to be tuned finely to maintain optimal cytosolic conditions for the performance of cellular functions.

The recent interest in vacuolar dynamics was sparked by the development of techniques for isolating intact mature vacuoles (Wagner & Siegelman, 1975; Leigh & Branton, 1976). This, together with the discovery of a marker for vacuolar membranes (tonoplast) - the anion-stimulated H^+-ATPase (Leigh & Walker, 1981) - allowed characterization of the biochemical properties associated with the vacuoles or with tonoplast vesicles obtained from tissue homogenates (Bennett *et al.*, 1984).

Vacuoles perform many functions that are essential for plant growth and survival. In meristematic cells, many small vesicles are observed which take up solutes and water, swell and coalesce to become a large central vacuole in the mature

cells, occupying more than 90 per cent of the intracellular volume (Owen & Poole, 1979). By increasing its volume the vacuole provides turgor and spreads the cytoplasm into a thin layer with a large surface area, thus allowing maximal exchange with the exterior and increased exposure to photosynthetically active radiation.

The vacuole has also been compared to the lysosomes in animal systems since many hydrolytic enzymes such as proteinases, acid phosphatase, RNase, DNase, ß-glucosidase among others are located within its boundaries (Matile, 1975). In addition, compounds potentially toxic to the cell (alkaloids, glycosides and phenolics) are accumulated, thus detoxifying the cytoplasm and even acting as an "arsenal of chemical weapons" against herbivores (Matile, 1987).

The bulk of the research performed on vacuoles and tonoplast vesicles in recent years is, however, related to transport mechanisms at the tonoplast, which regulate the exchange of solutes with the cytoplasm. This is the vacuole's homeostatic role, contributing to the regulation of cytoplasmic pH, Ca^{2+}, volume, etc., and acting as a temporary storage compartment for useful compounds. Some of the accumulated solutes include organic acids, amino acids, salts, sugars and nitrate (Boller & Wiemken, 1986).

The principles underlying solute transport across the tonoplast are based on Mitchell's chemiosmotic hypothesis (Mitchell, 1961) which links the proton gradients generated through redox reactions to the synthesis of ATP. In

mitochondrial or bacterial membranes, the energy obtained from the downhill translocation of protons by an ATP synthase is harnessed to phosphorylate ADP (Harold, 1986). At the tonoplast, on the other hand, the vacuolar ATPase (v-ATPase: EC 3.6.1.34), an enzyme similar to the mitochondrial F_1/F_O ATP synthase, but poised for the hydrolytic mode, pumps protons energetically "uphill" into the vacuole, at the expense of ATP (Sze, 1984). An electrochemical potential gradient $\Delta\mu_{H+}$ is thus generated across the vacuolar membrane. The energy provided by the chemical (ΔpH) and electrical ($\Delta\Psi$) gradients is utilized by a host of secondary transporters (antiports, symports, uniports) to drive the uptake of the various metabolites (Poole, 1988).

It was surprising, therefore, to find a second H^+-translocating enzyme, which utilized inorganic pyrophosphate (PPi) exclusively, located on the same membrane as the ATPase (Rea & Poole, 1985; Wang et al., 1986). This novel proton pump, the H^+-translocating inorganic pyrophosphatase (H^+-PPase; EC 3.6.1.1) has raised many questions about its physiological functions, regulation and phylogenetic origins.

This thesis is an attempt to answer some of these questions by initiating the molecular characterization of this newly discovered H^+-pumping enzyme.

CHAPTER 1

LITERATURE REVIEW

1.1. Plant H^+-translocating inorganic pyrophosphatases

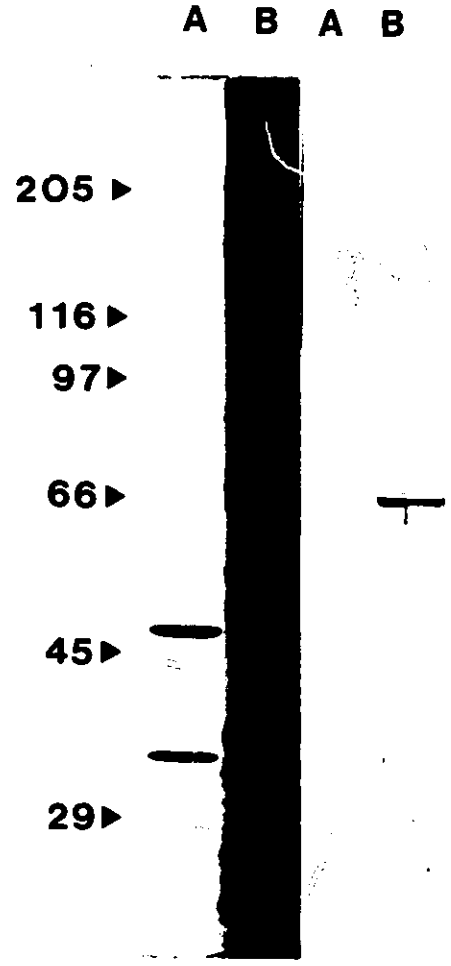
The existence of a membrane-bound inorganic pyrophosphatase in plants was first suggested by Karlsson (1975), who found it in the 25,000 to 30,000g fraction of sugar beet homogenates. Walker & Leigh (1981) later suspected the coupling of PPi hydrolysis with H^+ translocation on the basis of kinetic similarities between the plant PPase and the H^+-pumping PPase in Rhodospirillum rubrum (previously described by Klemme & Gest, 1971).

Chromatographic resolution of the H^+-translocating inorganic pyrophosphatase (H^+-PPase) from the vacuolar H^+-ATPase provided convincing evidence that two different proton pumps operated independently in the tonoplast to acidify the vacuole (Rea & Poole, 1986; Wang et al., 1986) and that it was not the H^+-ATPase which utilized PPi as a substrate. The distinctness of the H^+-PPase from soluble PPases was demonstrated by the lack of cross-reactivity between plant anti-H^+-PPase antibodies and purified yeast soluble PPase (Fig. 1.1).

Having been found in every plant thus far studied, the H^+-translocating inorganic PPase could well be ubiquitous in plant vacuolar membranes. It was identified in several crop plants such as Beta vulgaris storage roots (Walker & Leigh, 1981; Rea & Poole, 1985), Zea mays coleoptiles (Chanson et al., 1985), Triticum aestivum seedlings (Maslowski & Maslowska, 1987), Avena sativa roots (Wang et al., 1986)

Figure 1.1. **Immunoblot analysis of soluble PPase from yeast and of tonoplast from Beta vulgaris, probed with anti-H^+-PPase antibody from Vigna radiata.**

Left: Silver-stained SDS/PAGE of (A) 1 μg of commercially purchased (Sigma) purified yeast PPase and (B) 10 μg of tonoplast membranes from red beet roots. Right: Western blot of (A) 1 μg of purified yeast PPase and (B) 10 μg of beet tonoplast, probed with unfractionated antibody against mung-bean H^+-PPase. Arrows indicate molecular weight markers. Experimental details for Western blot as in section 2.3.8.


A B A B
205
116
97
66
45
29

and Vigna radiata hypocotyls (Maeshima & Yoshida, 1989), Tulipa petals (Wagner & Mulready, 1983), mesophyll protoplasts from the CAM plant Kalanchoe daigremontiana (Marquardt & Lüttge, 1987), thalli of the liverwort Riccia (Johannes & Felle, 1989), algae, such as Chara (Takeshige & Hager, 1988), Nitella (Shimmen & MacRobbie, 1987) and the unicellular marine alga Acetabularia (Ikeda et al., 1991).

Although animal endomembranes contain a vacuolar-type H^+-ATPase (Manolson et al., 1989), an equivalent H^+-PPase has not been identified. A recent report of a H^+-pumping PPase in vacuolar membranes of the yeast Saccaromyces carlsbergensis (Kulakovskaya et al., 1989) remains unconfirmed. No cross-reactivity was observed between anti H^+-PPase antibodies from mung-bean and S.cerevisiae vacuolar membranes (E.J. Kim, V. Sarafian and P.A. Rea, unpublished results). Moreover, PPi-energized H^+ pumping could not be recorded from S.cerevisiae vacuoles (M. Manolson, personal communication).

The existence of two proton pumping enzymes in the vacuolar membrane, prompts the question of simultaneous co-residence. Vacuoles from different developmental stages or tissue types could possess one pump type only. Unequivocal evidence that both pumps co-reside in the same cells came from isolated single vacuoles which were studied by either electrophysiological techniques (Hedrich et al., 1989) or direct visual observation with vital stains (Shimmen & MacRobbie, 1987).

1.2. Relative abundance of the plant H^+-PPase

Although the vacuolar H^+-PPase has been found in all plant systems thus far studied, its contribution to vacuole energization (relative to the H^+-ATPase) varies with plant species. Vacuolar vesicles isolated from mung-bean hypocotyls exhibited H^+-PPase specific activities four to six times higher than the H^+-PPase activity in the equivalent fraction of red beet roots (Sarafian & Poole, 1989; Maeshima & Yoshida, 1989). The purified enzyme in mung-bean represented approximately 10% of total vacuolar proteins (Maeshima & Yoshida, 1989). The contributions of the H^+-ATPases in beet and mung-bean were higher and lower respectively suggesting that the sum of the contributions of the two enzymes to the energization of the vacuole is more or less constant (Rea et al., 1992a). In the marine alga Acetabularia the H^+-PPase is approximately three-fold more active than the H^+-ATPase (Ikeda et al., 1991), whereas in Chenopodium rubrum suspension cells, the H^+-ATPase pumps protons at almost twice the rate of the H^+-PPase (Hoffman & Bentrup, 1989).

A comparison of three regions in mung-bean hypocotyls indicated that the H^+-PPase specific activities in the vacuolar membranes of dividing meristem cells, the elongating region and the mature region were not significantly different (Maeshima, 1990). There was however a ten-fold increase in vacuolar volume during the growth of the cells suggesting that there were more pumps per unit of vacuolar volume in the young cells than in the older ones. It would be tempting to

speculate that the PPase contributes directly to vacuolar expansion since it would raise the concentrations of translocated ions (especially if as suggested below, the PPase also transports K^+) to a higher level in a smaller volume and thus would permit a greater water uptake in the early stages of cell growth.

1.3. Kinetics of the H^+-PPase in plants.

In contrast to the anion-sensitive vacuolar H^+-ATPase, the H^+-PPase is cation-sensitive : K^+-stimulated, Na^+-inhibited. The concentration of K^+ for half-maximal stimulation of PPi hydrolysis ranges from 3.8 mM to 7 mM (White et al., 1990; Takeshige & Hager, 1988) and for half-maximal H^+-transport from 2.7 mM (White et al., 1990) to 19.8 mM (Takeshige & Hager, 1988). Na^+ could be acting as a competitive inhibitor (White et al., 1990).

The enzyme is completely dependent on Mg^{2+} for its activity, exhibiting complex kinetics when PPi and Mg^{2+} are varied independently. Complex formation by Mg^{2+} and PPi ($MgPPi^{2-}$, Mg_2PPi and $MgHPPi^-$) and pH-dependent variation of the PPi species (PPi^{4-}, $HPPi^{3-}$ or H_2PPi^{2-}) have confused the determination of the true substrate and how the other forms may compete for the active site. Some progress in the determination of the true substrate was recently made by White et al. (1990). Concentrations of each ionic species under saturating Mg^{2+} concentrations were computed with PPi concentrations as a variable. By correlating enzyme activity with the concentra-

tions of the different Mg/PPi complexes they determined that $MgPPi^{2-}$ was most likely to be the true substrate as it alone could support simple Michaelis-Menten kinetics. The K_m for PPi hydrolysis, with respect to $MgPPi^{2-}$ concentration was 17 μM and for H^+ transport the K_m value was 7 μM.

Many studies have also shown that the enzyme is dependent on the presence of free Mg^{2+} for activity. (Walker & Leigh, 1981; Marquardt & Lüttge, 1987). Having observed that increasing Mg^{2+} concentrations activate the enzyme in a sigmoid manner, White et al. (1990) suggested that the H^+-PPase is modulated through an allosteric mechanism and exhibits positive cooperativity with respect to Mg^{2+} binding. Hill coefficients for free Mg^{2+} activation of 2.5 and 2.7 for PPi hydrolysis and H^+ transport respectively indicated that there are at least three Mg^{2+} binding sites on the enzyme. It is not possible, however, to conclude from the Hill coefficients whether the enzyme has one subunit with three Mg^{2+} binding sites or several subunits with one site each (Segel, 1976).

1.4. H^+/PPi stoichiometry of the H^+-PPase

The existence of two pumps (both acidifying the vacuole) has prompted the still unanswered question of whether both work in vivo simultaneously, or if regulation by substrates and inhibitors causes them to function alternatively. Before attempting to answer this question, however, it should be established that the two pumps do indeed function in the same

direction (i.e., that they both energize the vacuole through substrate hydrolysis) and not in opposite directions, one in the hydrolytic and the other in the synthetic mode.

Based on a thermodynamic analysis of the putative Gibbs free energy changes accompanying ATP and PPi hydrolysis, Rea and Sanders (1987) confirmed that the ATPase would most likely translocate protons into the vacuole under physiological conditions, but left open the possibility that the PPase could be reversible. They calculated that only a stoichiometry of 1 H^+ translocated per PPi hydrolysed would allow hydrolysis to proceed at the transvacuolar pH gradients usually encountered (2 - 4 pH units). Stoichiometric ratios of 2 or 3 H^+/PPi would favor PPi synthesis: in this case the two pumps could function in opposite directions. It was crucial therefore to determine the H^+/PPi stoichiometry. Recent results from a thermodynamic study on Riccia intact vacuoles (Johannes & Felle, 1990) together with kinetic data from red beet tonoplast vesicles (Briskin, 1991) strongly suggest that the stoichiometry is $1H^+$/1PPi and that therefore the H^+-PPase is not likely to be reversible in vivo. Further evidence was obtained from patch-clamp studies on single vacuoles from sugar beet in which the H^+-PPase was shown to continue pumping protons even against a pH gradient of 4 units (Hedrich et al., 1989). Both pumps appear, therefore, to contribute to vacuolar acidification.

1.5. Inhibitors

One of the most commonly used inhibitors of the plant vacuolar H^+-PPase is F^-, which inhibits the enzyme with an I_{50} of 0.4 mM in Chara (Takeshige & Hager, 1988). Karlsson (1975) reported an inhibition reaching 97% with a 1 mM concentration of KF in sugar beet cotyledons. As F^- has no effect on the vacuolar ATPase or other membrane-linked phosphatases, it remains a powerful diagnostic tool for the identification of H^+-PPases. It is, however, equally effective against soluble PPases (Rip & Rauser, 1971).

Other, more specific PPase inhibitors are the substrate analogues imidodiphosphate (IDP) and methylenediphosphonate (MDP). These non-hydrolysable substrate analogs are thought to compete with PPi for the active site. The I_{50} value for IDP was in the range of 12 μM (Chanson & Pilet, 1988), whereas 1mM MDP inhibited the enzyme by 98.6% (Maslowski & Maslowska, 1987).

The sensitivity of the PPases to Ca^{2+} is documented in soluble (Moe & Butler, 1972) as well as membrane-bound PPases. In mung-bean, the complex CaPPi inhibits the vacuolar H^+-PPase with a K_i of 27 μm through competition with the substrate MgPPi (Maeshima, 1991). This value is close to the estimate of 29μM from the membrane PPase of Rhodopseudomonas palustris chromatophores (Schwarm et al., 1986). However, a recent study of the kinetics of Ca^{2+} inhibition of the H^+-PPase (Rea et al., 1992b) indicates that in vivo, it is more likely that free Ca^{2+}, at concentrations as low as 1 μM, is

the true inhibitor, acting through attachment to a site distinct from either the MgPPi or Mg^{2+} binding sites.

N-ethylmaleimide (NEM), which covalently binds sulfhydryl groups and inhibits the H^+-PPase with an I_{50} of 15 μM (Wang et al., 1986), has been successfully used for the characterization of the hydrolytic subunit of the H^+-PPase (Britten et al., 1989). Treatment of the tonoplast vesicles with ^{12}C-NEM in the presence of MgPPi, followed by incubation in ^{14}C-NEM in the absence of the substrate yields a single band on SDS-PAGE, indicating that the H^+-PPase has an essential, substrate-protectable sulfhydryl group. Inhibition of the H^+-PPase by N,N'-dicyclohexylcarbodiimide (DCCD; a strong inhibitor of the F-type and vacuolar H^+-ATPases) was only partial at 100μM (Wang et al., 1986; Maslowski & Maslowska, 1987).

The H^+-PPase is insensitive to inhibitors of other H^+ pumps such as vanadate, an inhibitor of plasma-membrane H^+-ATPase (Rea & Poole, 1985), oligomycin and azide, which inhibit the F_1F_O-ATPase (Rea & Poole, 1985, Wang et al., 1986), NO_3, an inhibitor of the vacuolar H^+-ATPase, and molybdate, an inhibitor of soluble phosphatases (Walker & Leigh, 1981; Rea & Poole, 1985).

1.6. Characteristics of the hydrolytic subunit

The hydrolytic subunit has been purified from red beet vacuoles and identified by correlation with activity (Sarafian & Poole, 1989), by ^{14}C-NEM labelling (Britten et al.,

1989) and by inhibition of activity with antibodies raised against the purified subunit from mung-bean (Maeshima & Yoshida, 1989). The molecular weight of the purified polypeptide, as estimated from SDS-PAGE, ranges from 64 kDa (Britten et al., 1989) and 67 kDa (Sarafian & Poole, 1989) in red beet to 73 kDa in mung bean hypocotyls (Maeshima & Yoshida, 1989). Although a value of 40 kDa has also been proposed in maize roots (Chanson & Pilet, 1989), this appears to be a breakdown product of the larger polypeptide as shown by Rea et al., (1992a).

The enzyme is composed exclusively of integral membrane subunits as shown by its lack of sensitivity to chaotropic agents such as high (0.6 M) KI or ClO_4^- concentrations (Rea et al., 1987). These cause the removal of peripheral polypeptides of other proteins such as the H^+-ATPase 57 kDa and 67 kDa subunits.

The gene encoding the hydrolytic subunit of Arabidopsis thaliana has been recently cloned and the amino-acid sequence determined (Sarafian et al., 1992b). The cDNA encodes a polypeptide of 81 kDa with at least 13 membrane spanning regions. The discrepancy between the size obtained from the cDNA and that from SDS-PAGE is probably due to anomalous migration of membrane proteins during SDS electrophoresis (Maddy, 1976).

Estimates of the functional sizes for substrate hydrolysis and H^+ transport have been obtained through radiation-inactivation studies on tonoplast vesicles isolated from

maize roots (Chanson & Pilet, 1989), Acer (Fraichard et al., 1991) and red beet roots (Sarafian et al., 1992a). The latter study suggests that a single catalytic subunit is sufficient to hydrolyze PPi but that the transport function requires an oligomer. Since the purified hydrolytic subunit can be reconstituted in a transport-competent form (Britten et al., 1992), it appears that the H^+-PPase holoenzyme is a homo-oligomer.

1.7. Physiological role of the H^+-PPase

Many cellular reactions produce pyrophosphate. Examples of such biosynthetic reactions are the amino-acylation of tRNA and the fatty-acylation of CoA which utilize ATP and release AMP and PPi (Stryer, 1981). Since the accumulation of products inhibits the forward reaction, it was generally assumed that the price to pay for maintaining the operation of synthetic reactions was the loss of the "high-energy bond" of PPi. The energy liberated by the hydrolysis of PPi was assumed to be dissipated as heat (Kornberg, 1963).

The available evidence, first observed in microorganisms (Wood, 1977) and confirmed in animals and plants (reviewed by Taiz, 1986) argues against the above hypothesis and supports the view that PPi utilization is, to a large extent, linked to energy-conserving reactions.

In plants, several observations imply coupling of biosynthetic processes with energy-conserving reactions. First, there seem to be no soluble PPases in the cytosol,

where the the bulk of the PPi is produced ; cytoplasmic PPi levels are in the range 0.2 to 0.3 mM in spinach leaves and represent about 90% of total cellular content (Weiner et al., 1987). In contrast, in the chloroplast, where soluble PPases have been identified (Bucke, 1970; Weiner et al., 1987), the concentration does not exceed 1 μM (Takeshige & Tazawa, 1989).

Second, several energy-conserving, PPi-dependent enzymes have been identified in the cytosolic compartment of plants. One such enzyme is the PPi-utilizing phospho-fructo-phospho-transferase (PPi-PFP) which is involved in the glycolytic pathway (Carnal & Black, 1983). PPi-PFP is regulated both by fructose 2,6-bisphosphate and PPi levels and its role in vivo is still unclear since an ATP-dependent phosphofructokinase already exists which performs the same function of phospho-rylating fructose 6-phosphate in the glycolytic cycle. Some studies suggest that PPi-PFP might be more active in situ-ations of stress which would lower ATP levels such as anaero-biosis (Mertens et al., 1990) or in cells which are very active metabolically, since increases in PPi-PFP levels seem to correlate with regions of cell division and expansion (Stitt, 1990). Another energy-coupled enzyme is the vacuolar H^+-translocating inorganic PPase whose hydrolytic active site is located on the cytosolic side of the tonoplast.

Third, the PPi concentrations in the cytosol are suffi-cient to drive the above coupled enzymes at maximum rates, since cytoplasmic PPi levels appear to be relatively constant

at about ten times the K_m value of either enzyme (Stitt, 1990).

The energy-conserving reactions catalyzed by the H^+-PPase have demonstrated that the enzyme plays a physiological role far more important than simply reducing PPi levels in the cytoplasm (Rea & Sanders, 1987). Acidification of the vacuole by the PPase's H^+-translocating function can, in some plants, supersede that attributed to the H^+-ATPase residing on the same membrane (Johannes & Felle, 1990). In situations of diminishing ATP/ADP ratios, such as during Pi limitation (Dancer et al., 1990) or anaerobiosis (Mertens et al., 1987), the presence of the H^+-PPase could ensure that vital functions related to ionic balance between the cytoplasm and the vacuole are not adversely affected.

In addition, a new function has been tentatively ascribed to this enzyme: the translocation of K^+ with H^+ into the vacuole (Davies et al., 1991). This property (recently identified by calculations of the effect of K^+ on the reversal potential of the pump) would provide the H^+-PPase with a role in the accumulation of K^+ as a major osmoticum in the vacuole. That function awaits a direct demonstration of PPi-dependent K^+ uptake. At present, no convincing argument has been presented for the accumulation of K^+ in the vacuole other than through non-specific cation channels (Hedrich et al., 1986; Pantoja et al., 1989). Given the observed positive-inside potential of vacuoles of about 20 - 50 mV (Spanswick & Williams, 1964; Bates et al., 1982), a passive

equilibrium of K^+ between the cytoplasm and the vacuole would result, according to the Nernst equation, in vacuolar K^+ concentrations between one third and one tenth of that in the cytoplasm. Since measurements of K^+ in the vacuole and in the cytoplasm yield approximately equal concentrations in the two compartments (Leigh & Wyn Jones, 1984; Malone et al., 1991), it is very likely that energized K^+ transport occurs at the tonoplast. Such a system would be required to explain the swelling of guard cells during stomatal opening and of motor cells of pulvini, where large amounts of K^+ are accumulated in the vacuole causing water uptake, without much change in cytoplasmic volume (Campbell & Garber, 1980; Gorton, 1990). Transport of K^+ by the vacuolar H^+-PPase would mean that this enzyme could (at least partially) regulate stomatal and pulvinar movement and possibly play a substantial role in turgor regulation which is achieved largely by ion movements into and out of the vacuole.

1.8. Regulation of H^+-PPase activity

Factors regulating the H^+-PPase activity have not been clearly determined yet, but from the PPi concentrations measured in the cytosol, the enzyme does not seem to be substrate regulated. Mg^{2+} has been suggested as a possible regulator (White et al., 1990) since the available pool has to be shared with other Mg^{2+}-binding compounds such as ATP. Cytoplasmic free Mg^{2+} concentrations are about 0.4 mM in mung-bean but increase to 0.68 mM when ATP levels drop (Yazaki et

al., 1988). In this range the H^+-PPase would be Mg^{2+}-regulated and its activity inversely related to that of the H^+-ATPase.

Regulation by Ca^{2+}, which inhibits the enzyme at micromolar concentrations (Rea et al., 1992b) is another possibility which deserves attention , especially in light of recent findings of Ca^{2+} release into the cytoplasm of Commelina guard cells immediately preceding stomatal closure (McAinsh et al., 1990; Gilroy et al., 1991). Increases in cytoplasmic calcium levels have been observed when abscisic acid, which causes stomatal closure, is applied to Vicia faba guard cells (Schroeder & Hagiwara, 1990). Calcium appears to be mobilized from internal stores, most probably from the vacuoles (Gilroy et al., 1990) resulting in the regulation of ion channels at the plasma-membrane (Schroeder & Hagiwara, 1989) and the vacuole (Hedrich & Neher, 1987) and, in all likelihood, inhibition of the H^+-PPase.

1.9. H^+-PPases in photosynthetic bacteria

The first documentation of a proton-coupled PPi hydrolyzing enzyme was in the photosynthetic bacterium Rhodospirillum rubrum (Baltscheffsky et al., 1966). This membrane-bound PPase was located in the chromatophores and was easily reversed to the synthetic mode on illumination. Indeed, PPi synthesis was suggested to be its primary function in the bacteria (Behrens & De Meis, 1985; Nore et al., 1986).

The H^+-PPase in Rhodospirillum has recently been isolated and found to consist of a single polypeptide of 56 kDa

on SDS gels and to represent about 1% of the proteins in chromatophores (Nyrén et al., 1991). Radiation inactivation studies suggest an oligomeric structure for the functional enzyme of 168 kDa and 153 kDa for its hydrolytic and H^+-pumping activities respectively (Wu et al., 1991). Although many of the bacterial enzyme's characteristics are similar to those of the plant H^+-PPase, such as its requirement for free Mg^{2+}, its sensitivity to the same inhibitors and its extremely hydrophobic nature, two major differences suggest they fulfill functionally distinct roles. First, it is located on the plasma membrane of the bacterial cell (chromatophores are plasma membrane-derived vesicles - Nyrén et al., 1991) and its primary role appears to be the light-driven synthesis of PPi, which can subsequently be used in the production of ATP (Keister & Minton, 1971). The H^+-PPase in R. rubrum is also readily reversible (Nyrén & Baltscheffsky, 1983). The second major difference is its lack of requirement for K^+, which is essential for activation in the plant enzyme.

Structural similarities between the plant and bacterial enzymes will have to await determination of amino-acid sequences but recent investigations of the immuno-reactivity between plant antibodies and the bacterial enzyme show recognition of the bacterial antigen by plant H^+-PPase antibodies (Nore et al., 1991). Another H^+-PPase has recently been identified in chromatophores of the photosynthetic bacterium Rhodopseudomonas palustris and an initial characterization indicates that it is very similar to the Rhodospirillum

enzyme (Schwarm et al., 1986).

1.10. Mitochondrial pyrophosphatases

Two different forms of mitochondrial pyrophosphatases, reviewed by Mansurova (1989), have been isolated from rat liver mitochondria (Volk & Baykov, 1984), beef heart mitochondria (Volk et al., 1983) and yeast mitochondria (Lundin et al., 1991). Both of these types, one soluble and the other membrane bound, are found in all three organisms, suggesting a universal occurrence although only the membrane bound type has been studied in plants (Kowalczyk and Maslowski, 1981; Vianello et al., 1991). The membrane-bound PPase is competent in H^+ translocation (Mansurova, 1989; Vianello et al., 1991) but does not appear to be related structurally to the vacuolar enzyme. In liver mitochondria, it is made up of four polypeptide species and has been tentatively assigned the structure $\alpha\beta\gamma_2\delta$. Its molecular mass is about 185 kDa and it is located on the inner side of the inner membrane to which it is loosely bound, being easily removed by ultrasonic treatment or with 0.25M sucrose. The soluble enzyme is found in the matrix, is a dimer ($\alpha\beta$) of about 60 kDa and has very similar kinetic and inhibitor characteristics to the membrane-bound form (Mansurova. 1989).

The catalytic subunit of the yeast mitochondrial membrane-bound PPase has recently been cloned and its sequence elucidated (Lundin et al., 1991). The high homology with the cytoplasmic enzyme from the same organism (49%), suggests

that the mitochondrial PPase is more closely related to soluble PPases than to the vacuolar type.

1.11. Soluble pyrophosphatases

Enzymes which hydrolyse PPi are found in all living organisms and have been thoroughly studied since the early 1950's (Lahti, 1983). The most extensively studied PPases are those from E. coli and Saccaromyces cerevisiae, whose genes have been isolated and the amino-acid sequences obtained. The three-dimensional structure of the yeast enzyme has been determined from X-ray diffraction and this PPase is now commercially available in purified form.

The soluble PPases are smaller than the membrane-bound counterparts, with subunit sizes ranging from 20 kDa in E. coli (Lahti et al., 1988), to 32 kDa in yeast (S. pombe is 32.5 kDa ; Kawasaki et al., 1990) and 40 kDa in sorghum chloroplasts (Krishnan & Gnanam, 1971), but 30 kDa in Arabidopsis (Kieber & Signer, 1991). No PPases have been isolated from plant cytosolic fractions, the enzyme being confined to the chloroplast. The enzymes differ in the number of subunits making up the holoenzyme, the bacterial type being composed of homohexamers, whereas yeast and plant types are made up of homodimers and monomers respectively.

The kinetics of activation and inhibitor sensitivities are very similar among all organisms: low micromolar range of K_m for substrate hydrolysis, inhibition by free PPi and additional binding sites for Mg^{2+}, which is a required activator

(Springs et al.,1981; Lahti, 1983). One major difference from vacuolar H^+-PPases is the lack of dependence on K^+ for activity.

1.12. Summary

The H^+-PPase is a novel H^+-translocating enzyme, located at the tonoplast, which together with the H^+-ATPase energizes the vacuole. It is also active in the breakdown of PPi generated by anabolic reactions in the cytosol. Its low K_m, relative to the concentration of the substrate ($MgPPi^{2-}$), suggests that it could function at V_{max} under most physiological conditions. It could be allosterically regulated by free Mg^{2+}, which binds the enzyme at 3 or more sites and/or by Ca^{2+} which causes inhibition at the low micromolar concentrations often encountered in vivo.

Harsh treatment of the H^+-PPase with chaotropic agents does not affect its hydrolytic or H^+ pumping activities, indicating that the enzyme is intrinsically associated with the membrane. In CHAPTER 2 it is shown that, in red beet vacuoles, the enzyme has only one subunit species with a size of about 67 kDa as determined from SDS/PAGE. CHAPTER 3 examines the minimum functional sizes of the H^+-PPase for PPi hydrolysis and for H^+ translocation. This is accomplished through the technique of radiation inactivation on red beet tonoplast vesicles. From this study it is concluded that, for substrate hydrolysis, each subunit is functionally independent but that a minimum of three subunits are required for

the H^+-pumping function.

Structural similarities with known plant H^+-ATPases (P-, F- and V-type) can be excluded on the basis of subunit composition, inhibitor sensitivity and substrate specificity. It is, however, more difficult to exclude phylogenetic links between the plant H^+-PPase and other known PPases since, despite considerable size differences, they share common kinetic characteristics. In CHAPTER 4, the isolation of a cDNA encoding the H^+-PPase gene from Arabidopsis is reported. The deduced sequence shows no homologies with soluble PPases from bacteria or yeast, confirming the novelty of this enzyme. The most likely candidate for phylogenetic similarity, however, the H^+-PPase from photosynthetic bacteria, awaits elucidation of its primary sequence.

CHAPTER 2

Purification of an H^+-translocating inorganic pyrophosphatase from vacuole membranes of red beet

2.1. ABSTRACT

An H^+-translocating inorganic pyrophosphatase (H^+-PPase) was isolated and purified from red beet (Beta vulgaris L.) tonoplast. One major polypeptide of molecular weight 67 kDa copurified with fluoride-inhibitable PPase activity when subjected to one dimensional polyacrylamide gel electrophoresis. Overall, a 150-fold purification of the H^+-PPase was obtained, from the tonoplast fraction, through anion exchange chromatography of the detergent-solubilized membranes followed by ammonium sulfate precipitation and gel filtration chromatography. The purified polypeptide showed no cross-reactivity with antibodies raised against the 67 kDa subunit of the tonoplast H^+-ATPase.

2.2. INTRODUCTION

The existence of two distinct H^+-translocating pumps, an H^+-ATPase and an H^+-pyrophosphatase (H^+-PPase), both potentially contributing to the electrochemical gradient across the vacuolar membrane, has now been well documented. The enzymes have been shown to be separable by chromatography after detergent-solubilization of tonoplast vesicles isolated from red beet (Rea and Poole, 1986), and from the CAM plant Kalanchoe daigremontia (Bremberger et al., 1988). Both proton pumps caused accumulation of neutral red in individual vacuoles of Nitella (Shimmen and MacRobbie, 1987) and in a patch-clamp study of sugar beet vacuoles, Hedrich and Kurkdjian (1988) demonstrated the presence of the two electrogenic pumps, by measuring membrane potentials induced by ATP and/or PPi in the same vacuole.

As a first step towards a more complete molecular analysis of the protein, it was necessary to purify the H^+-PPase and elucidate its polypeptide composition. A recent study which reported the purification of the H^+-PPase from microsomal fractions of corn seedlings (Maslowski and Maslowska, 1987) could not, however, be applied to our system of purified tonoplast from red beet and we have, therefore, developed an alternative purification scheme.

2.3. MATERIALS AND METHODS

2.3.1. Tonoplast preparation.

Fresh red beets (Beta vulgaris L.) were bought commercially, kept at 4°C and used within 24 hours.

Tonoplast vesicles were prepared as described (Rea and Poole, 1985) with some modifications. Peeled and diced beets (350 g) were homogenized at 4°C in a blender with two 30-second bursts in 350 mL homogenization buffer containing 10 mM glycerophosphate, 0.65 M ethanolamine (adjusted to pH 8.0 with concentrated H_2SO_4), 0.28 M choline chloride, 26 mM K-metabisulfite, 3 mM BTP-EDTA, 0.2% BSA (fraction V, essentially fatty acid-free), 10% (w/v) insoluble PVP, 5 mM DTT and 1 mM PMSF in 70 mM BTP-Mes pH 8.0. The homogenate was filtered through cheesecloth and centrifuged at 80,000 g for 30 minutes in a Beckman Type 45 Ti rotor. The pellets were resuspended with a Dounce homogenizer in a small volume of suspension medium (SM) containing 10% (w/v) glycerol, 5 mM BTP-Mes pH 8.0, 1 mM BTP-EDTA and 5 mM DTT, then made up to 50 mL, mixed with an equal volume of 0.8 M KI and centrifuged as above. The KI-treated pellets were resuspended in SM, homogenized, and 7.5 mL aliquots layered on 3 step gradients of 10 mL 10% sucrose (w/w) and 20 mL 23% sucrose (w/w) in SM. After centrifugation at 80,000 g for two hours in a Beckman SW 28 rotor, the 10/23% interfaces were collected, diluted 7-fold with SM and sedimented at 80,000 g for 30 min. The pellets were resuspended in 2-3 mL of the same solution and stored at -70°C.

The identification of the 10/23% sucrose gradient interface as the tonoplast fraction has already been documented (Rea and Poole, 1985).

2.3.2. Solubilization.

Tonoplast aliquots (6-8 mg protein) were pelleted at 80,000 g for 30 min in a Beckman SW 60 rotor and the pellets resuspended in 2 mL of a solution containing 2.5% Triton X-100, 10% (w/v) glycerol, 30 mM BTP-Mes pH 8.0, 4 mM MgCl2, 1 mM BTP-EDTA and 5 mM DTT. After 5-6 strokes in a Dounce homogenizer, the mixture was incubated on ice for 1 hour on an orbital shaker at 100 rpm and homogenized again. The supernatant from a 200,000 g spin for 40 min in the same rotor was assumed to contain the solubilized enzyme.

2.3.3. PPase and ATPase activities.

PPase and ATPase activities were measured by the method of Ames (1966), as described (Rea and Poole, 1985). Phospholipids were prepared from partially purified soybean phospholipids (Type IV from Sigma) by sonication (Manolson et al., 1985).

2.3.4. Anion-exchange Chromatography.

A 1.5 cm x 10 cm column was filled with 20 mL DEAE-Sephacel (Pharmacia Canada Ltd.), washed with 50 mL of 1 M KCl, followed by 50 mL of 0.25 M His/Cl pH 6.0 and finally equilibrated with an elution buffer (EB) containing 20 mM

His/Cl pH 6.0, 10% (w/v) glycerol, 0.25% Triton X-100, 40 mM KCl, 2 mM $MgCl_2$ and 5 mM DTT.

Two mL of solubilized tonoplast was diluted 10-fold with EB without Triton X-100 (to equalize detergent concentrations) but with 0.5 mg/mL phospholipid and the sample was loaded on the column at a rate of 20 mL/hr, followed by a wash with 10 mL EB + 0.5 mg/mL phospholipid. Proteins were eluted with 50 mL of a linear KCl gradient (40 mM to 300 mM in EB + 0.5 mM phospholipid) and collected in 1.6 mL fractions in test-tubes pre-loaded with 100 µL EB + 8 mg/mL phospholipid. The column was regenerated with 1 M KCl.

2.3.5. Ammonium sulfate precipitation.

Fractions from the anion-exchange column containing the highest PPase activities were pooled and sufficient $(NH_4)_2SO_4$ was added to bring the solution to 30% saturation and mixed. After incubation on ice for 10 minutes (shaking), the mixture (6-8 mL) was centrifuged at 10,000 g for 10 minutes in a Sorvall RC-5. The supernatant was aspirated off and the pellet resuspended in 0.8 mL of gel filtration running buffer.

2.3.6 Gel filtration chromatography.

A 1 cm x 100 cm column was packed with Sephacryl S400HR (Pharmacia Canada Ltd.) at 60 mL/hr and equilibrated with running buffer (RB) containing 1% CHAPS, 30 mM BTP-Mes pH 8.0, 10% (w/v) glycerol, 25 mM KCl, 4 mM MgCl2, 1 mM BTP-

EDTA, 10 mM DTT, 0.03% (w/v) NaN_3 and 2.0 mg/mL phospholipid. Between 0.1 and 0.2 mg protein from the $(NH_4)_2SO_4$ purified fraction was applied to the column and eluted with RB at 5 mL/hr at 2°C.

2.3.7 Preparation of samples for SDS-PAGE.

Fractions (1.25 mL) eluted from the gel filtration column were assayed for PPase activity, pooled in groups of three and precipitated in 15 mL Corex tubes with ice cold TCA at a final concentration of 15%. After a 30 minute incubation on ice, the samples were spun at 10,000 g for 15 minutes and the supernatants aspirated off. Twenty microliters of sample buffer (10% glycerol, 10% SDS, 100 mM DTT, 63 mM Tris-Cl, pH 6.8 and bromphenol blue) diluted five-fold were added to each tube and the pellets resuspended by thorough vortexing and swirling. Since enough acid usually remained at the bottom of the tubes to turn the bromphenol blue yellow, small aliquots (2-3 μL) of 1 M Tris were added to the tubes and swirled until the tubes turned blue. The samples were transfered to 1.5 mL microfuge tubes and stored overnight at 4°C, the volumes were then reduced to approximately 20 μL by vacuum centrifugation (Speed-Vac, Savant Instruments) and electrophoresed directly.

2.3.8 SDS-PAGE and Western blot.

One-dimensional SDS-PAGE was performed as described by Laemmli (1970). Ten percent acrylamide gels were run in a

Mini-Protean apparatus (Bio-Rad). Protein was detected by Coomassie Blue staining. Molecular weight standards were purchased as a kit (High MW Standards - Sigma).

Western blotting to a nitrocellulose filter was carried out in a Mini Trans-Blot apparatus (Bio-Rad). After the transfer, the filter was blocked for 1 hour in TTSB (0.07% Tween 20, 0.9% NaCl, 10 mM Tris/Cl pH 7.3) containing 5% powdered skim milk. After 3 x 5 min rinses in TTSB, the filter was incubated for 6 h in antibody (1/300 dilution with TTSB). After rinsing as above, the filter was incubated for 2 h in TTSB containing HRP-conjugated goat anti-rabbit IgG (Bio-Rad). After rinsing in TSB (TTSB without Tween 20), immunoreactive bands were visualized with H_2O_2/4-chloro-1-naphthol.

2.3.9 Densitometry.

An Ultrascan XL Laser Densitometer (LKB) was used to obtain quantitative density profiles of the Coomassie stained acrylamide gels.

2.3.10 Protein.

Protein was measured by the method of Lowry et al., (1951), after treatment of the samples by the method of Wessel and Flügge (1984).

2.3.11 Reagents.

Sephacryl S400HR and DEAE-Sephacel were purchased from

Pharmacia (Canada) Ltd., CHAPS from Pierce Chemical Co., DTT and Histidine from ICN Canada Ltd., and Mes from Boehringer Mannheim Canada Ltée. All other reagents were purchased from either Sigma Chemical Co. or BDH Chemicals, Canada.

2.4. RESULTS

2.4.1. Anion-exchange chromatography.

Six to 8 mg of solubilized membranes were loaded onto the DEAE - Sephacel column and eluted with a linear KCl gradient (40 mM to 300 mM) as described in "Materials and Methods". Figure 2.1 shows that the H^+-PPase eluted in a sharp peak between 80 mM and 120 mM and was separated from the ATPase activity which eluted between 200 mM and 250 mM KCl. Phospholipids had to be present at all stages in order to prevent denaturation of the H^+-PPase. Since phospholipids appeared to bind the column and elute later than the H^+-PPase, it was essential to have additional phospholipids in the tubes where the fractions were collected.

The anion-exchange chromatography was performed at pH 6.0. Initial attempts to run the column at pH 8.0, the optimal pH for activity of the enzyme, resulted in almost complete irreversible inactivation (1.4% activity recovered). At pH 7.0, 27% activity was recovered and at pH 6.0, 100% of the PPi hydrolytic activity applied was accounted for. The enzyme was always assayed at pH 8.0.

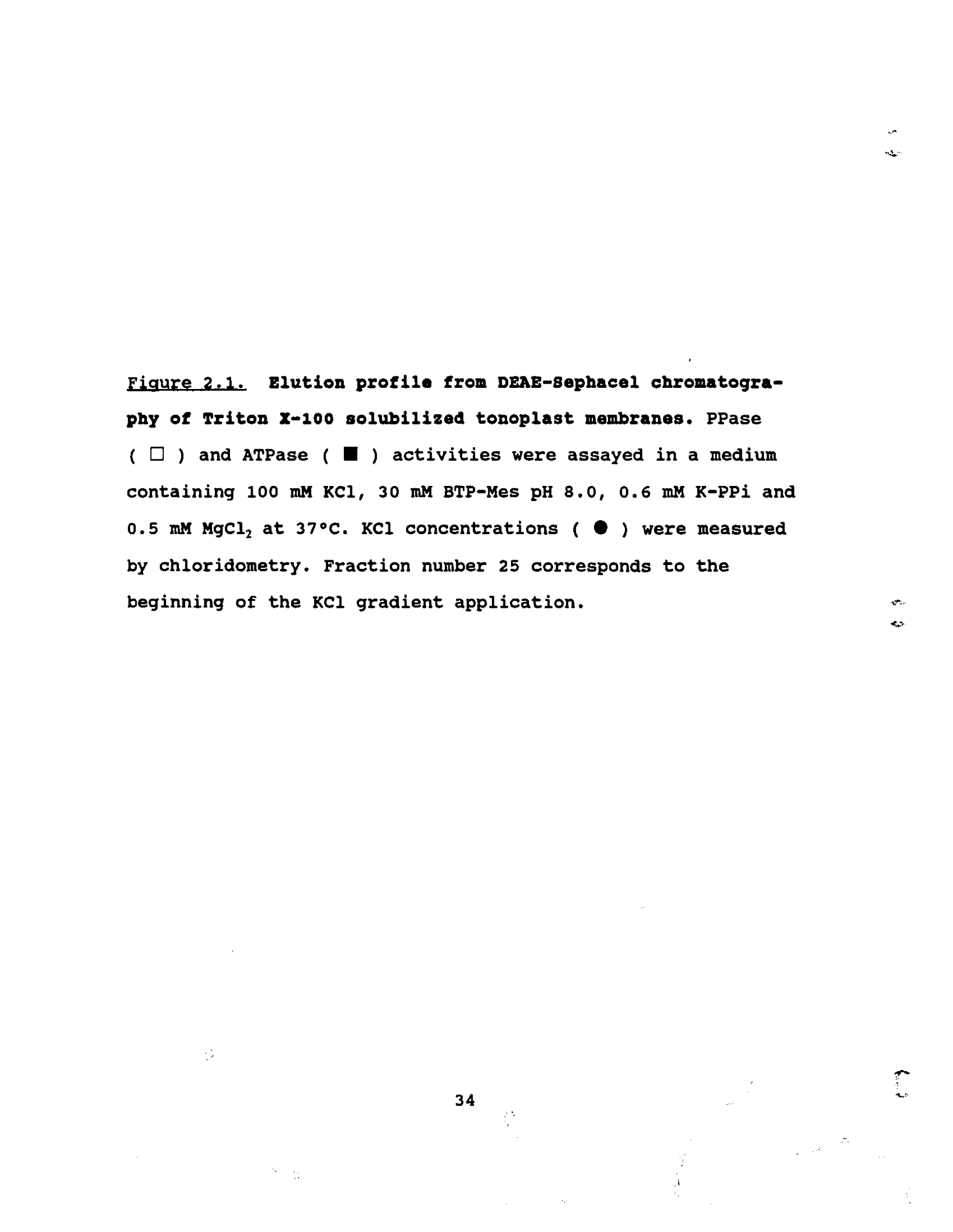
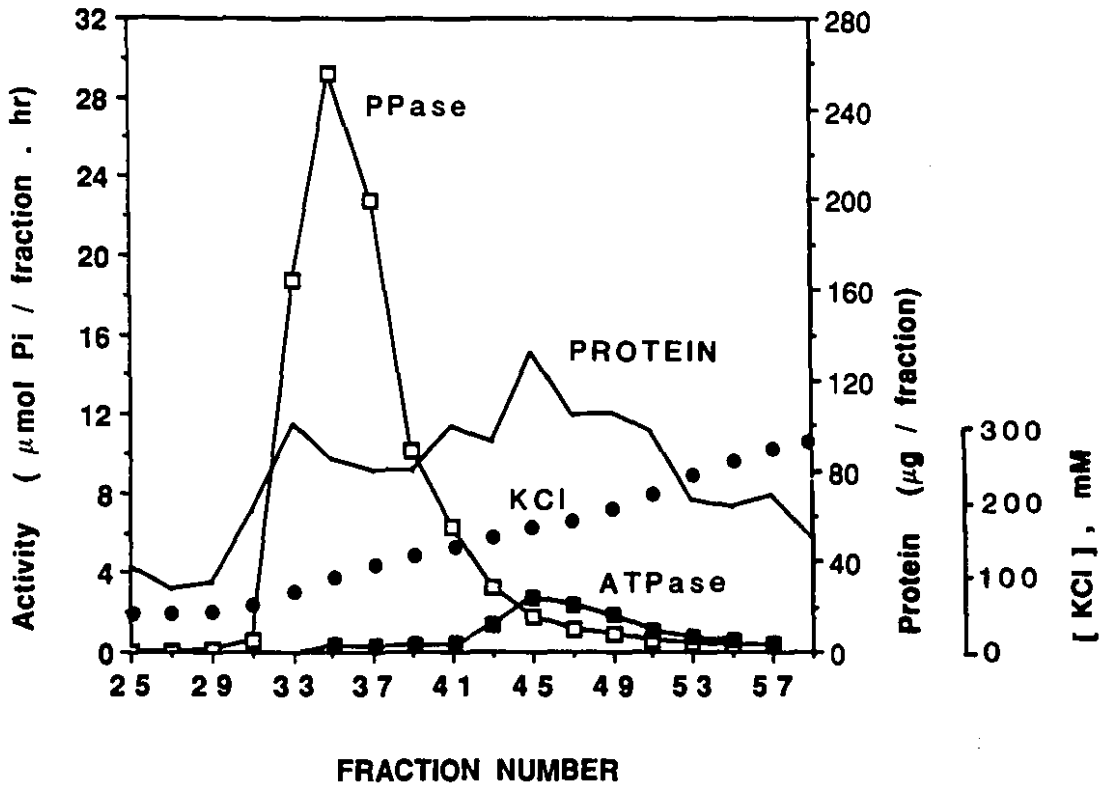


Figure 2.1. **Elution profile from DEAE-Sephacel chromatography of Triton X-100 solubilized tonoplast membranes.** PPase (□) and ATPase (■) activities were assayed in a medium containing 100 mM KCl, 30 mM BTP-Mes pH 8.0, 0.6 mM K-PPi and 0.5 mM $MgCl_2$ at 37°C. KCl concentrations (●) were measured by chloridometry. Fraction number 25 corresponds to the beginning of the KCl gradient application.


PPase
PROTEIN
KCl
ATPase
Activity (μmol Pi / fraction . hr)
Protein (μg / fraction)
[KCl], mM
FRACTION NUMBER
0
4
8
12
16
20
24
28
32
0
40
80
120
160
200
240
280
0
100
200
300
25
29
33
37
41
45
49
53
57

2.4.2. Ammonium sulfate precipitation.

After anion-exchange chromatography, $(NH_4)_2SO_4$ precipitation was used to further purify and concentrate the enzyme. With $(NH_4)_2SO_4$ at a saturation level of 30%, about one third of the protein and 50% of the PPase activity were precipitated (Table 2.1).

2.4.3. Inhibitor sensitivity.

Sensitivity of the PPi hydrolysis to the PPase inhibitor F- (Rip and Rauser, 1971 ; Karlsson, 1975) was measured on the solubilized fraction and after the final purification step of the H+-PPase. Residual activity after treatment with 10 mM KF of the solubilized and purified enzymes were 26.3 and 15.4 percent respectively, confirming that the purified phosphohydrolytic activity was that of the H+-PPase.

2.4.4. Gel filtration on Sephacryl S400HR.

A further ten-fold increase in specific activity was obtained in the peak H+-PPase fractions after gel filtration (Table 2.1). This additional purification was made possible by the replacement of Triton X-100 with CHAPS in the running buffer which resulted in the relatively earlier elution of the H+-PPase giving a better separation from other proteins. It was also found that having CHAPS in the samples applied to the SDS-PAGE produced less distortion of the bands than Triton X-100.

Table 2.1. Balance sheet of H+-PPase purification from red beet tonoplast.

Tonoplast vesicles were solubilized in 2.5% Triton X-100 and subjected to DEAE-Sephacel chromatography. Peak PPase containing fractions were combined and precipitated with $(NH_4)_2SO_4$ up to a saturation level of 30%. The pellet was resuspended in 0.8 mL of gel filtration running buffer and applied to a Sephacryl S400HR column. Eluting fractions were assayed for PPase activity, grouped in three's and subjected to SDS-PAGE. Lane 4 refers to Figure 2.2. Protein in lane 4 was estimated by comparing staining densities of the bands with those of the molecular weight standards whose concentrations were known.

	Protein		PPase activity		
	mg	%	µmol/hr	%	µmol/mg.hr
Tonoplast	6.98	100.0	93.0	100.0	13.3
Triton X-100 solubilized	4.62	65.7	160.3	172.3	34.7
DEAE-Sephacel: total	3.05	43.6	157.6	169.5	51.7
" peak fractions	0.66	9.4	85.9	92.4	130.2
Ammonium sulfate	0.23	3.3	43.1	46.3	187.4
Sephacryl S-400: total	N/Da	-	21.0	22.6	-
Peak (lane 4 of Fig 2.2)	≈ 0.004	≈ 0.06	8.1	8.7	≈ 2000

N/Da = not determined

2.4.5. Balance sheet.

The various purification steps resulted in an approximately 150-fold purification of the H+-PPase from the KI treated tonoplast membranes. The specific activity increased from 13.3 µmole Pi.mg-1.hr-1 to near 2000 µmole Pi.mg.-1.hr-1 (Table 2.1). The protein estimate in the peak fraction was made by comparing the staining density on the SDS gel with the molecular weight standards whose concentrations were known.

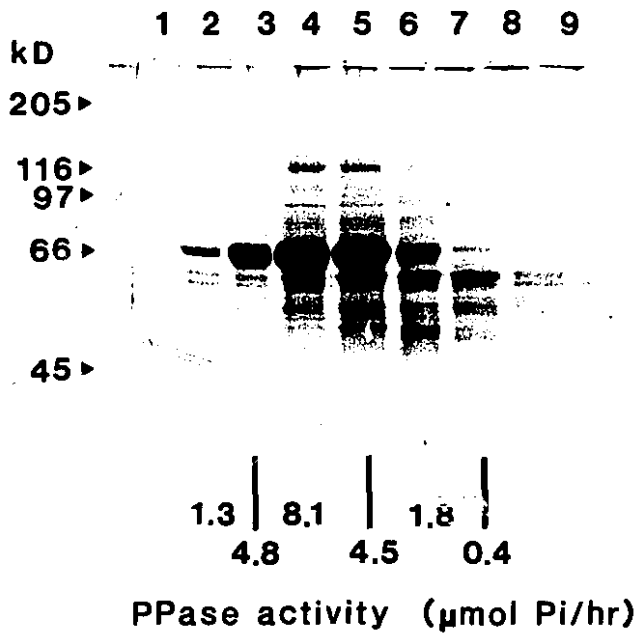
2.4.6. SDS/PAGE.

Electrophoresis of the fractions collected from the gel filtration step revealed a polypeptide of molecular weight 67 kDa which copurified with the PPase activity (Fig. 2.2). A correlation coefficient of r = 0.97 was obtained between the enzyme activities in each lane and the density of the 67 kDa band, determined by densitometry. Other bands, such as those around 115 kDa, or at 60 kDa showed weak correlations with activity ; they did not, however, consistently copurify with PPase activity.

2.4.7. Western blot.

The association of PPase activity with a 67 kDa polypeptide raised the possibility that this activity could be due to the subunit of the ATPase of the same size (Manolson et al., 1985) which after passage through the

Figure 2.2. **Identification of the 67 kDa polypeptide by correlation with PPase activity.** Fractions (1 mL) collected from Sephacryl S400HR chromatography were assayed for PPase activity, combined in groups of three and subjected to SDS-PAGE (lanes 1 - 9). Numbers at the bottom represent total PPase activities in the fractions applied to the corresponding lanes. Molecular weight standards are shown on the left. Staining densities were quantified by densitometry and correlated with enzyme activities.


1 2 3 4 5 6 7 8 9
kD
205
116
97
66
45
1.3
8.1
1.8
4.8
4.5
0.4
PPase activity (µmol Pi/hr)

anion-exchange column could conceivably be modified to hydrolyze PPi. To examine that possibility, an immunodetection assay was performed against a Western blot from an SDS-PAGE of the fractions eluted from the anion-exchange column. Antibodies raised previously against the 67 kDa subunit of the ATPase (Manolson et al., 1989) showed no cross-reactivity with polypeptides from the fractions where the H+-PPase was found (Fig 2.3).

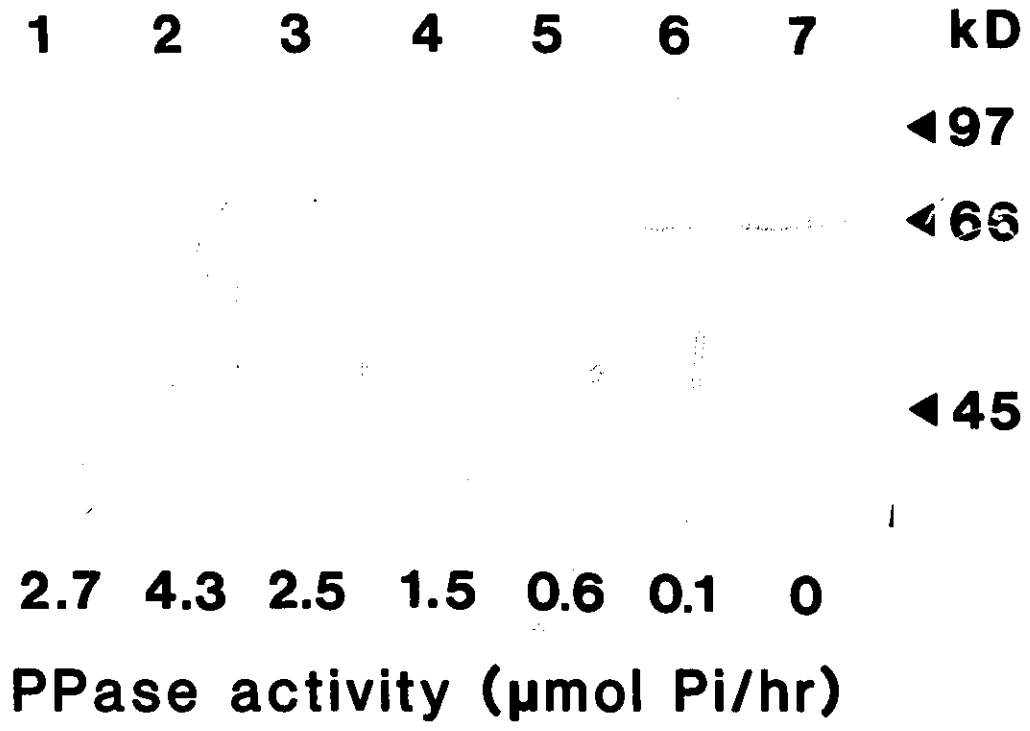
2.5. DISCUSSION

Purification of the H+-translocating inorganic PPase from red beet vacuolar membranes indicates that the catalytic activity of this enzyme is associated with one polypeptide species of 67 kDa. This activity is inhibited by F-, a PPase inhibitor. The estimated molecular weight of the H+-PPase is close to that of the 64 kDa polypeptide isolated from corn seedling microsomal membranes (Maslowski and Maslowska, 1987). The origin of their enzyme, however, appears uncertain since they collected their membranes from a 33/46% sucrose interface, where most cell membranes are found. Among other H+-coupled PPases so far characterized, the photosynthetic bacterium Rhodospirillum rubrum has been tentatively assigned two subunits of 54 kDa and 52 kDa (Baltscheffsky and Niren, 1987) ; from rat liver mitochondrial PPase II, two subunits of 28 kDa and 35 kDa have been identified (Volk and Baykov, 84) and from bovine heart mitochondria subunits of 28 kDa, 30

Figure 2.3. **Absence of cross-reactivity between anti-ATPase (67 kDa) antibody and H+-PPase hydrolytic subunit.***

Successive fractions from a DEAE-Sephacel column (corresponding to fractions 30 to 50 of Fig. 2.1) were pooled in groups of three and small aliquots subjected to SDS/PAGE, followed by electrophoretic transfer to nitrocellulose (Western). The blot was probed with antibodies against the 67 kDa subunit of vacuolar ATPase and visualized with peroxidase-coupled goat anti-rabbit IgG (Bio-Rad).

* This figure was reported as "data not shown" in the published version.


1 2 3 4 5 6 7 kD
◀97
◀66
◀45
2.7 4.3 2.5 1.5 0.6 0.1 0
PPase activity (μmol Pi/hr)

kDa, 40 kDa and 60 kDa have been reported (Volk et al., 1983). In the latter enzyme, the authors suggest that the catalytic activity resides in the smaller subunits and therefore no significant similarities would be expected, based on subunit size between red beet tonoplast H+-PPase and other membrane-bound PPases. As for soluble PPases, their subunit sizes range from 42 kDa in sorghum chloroplasts (Krishnan and Gnanam, 1988) to 19.5 kDa in E. coli (Lahti et al., 1988) and any similarities with the membrane type H+-PPases will have to await more detailed structural analyses of these polypeptides. Although we cannot yet predict with accuracy the size of the holoenzyme, an approximate size of the protein-detergent-lipid complex of 250 ± 50 kDa has been estimated from the gel filtration experiments.

Membrane preparations were treated with KI to reduce contamination by acid phosphatases (Poole et al, 1984). As well, Lai et al (1988) showed that in oat roots, 75% of ATPase activity and approximately 35% of total membrane protein was removed by 0.4 M KI. We did not observe any concommitant loss of PPase activity in red beet. This step was therefore routinely used to increase the specific activity of the starting material.

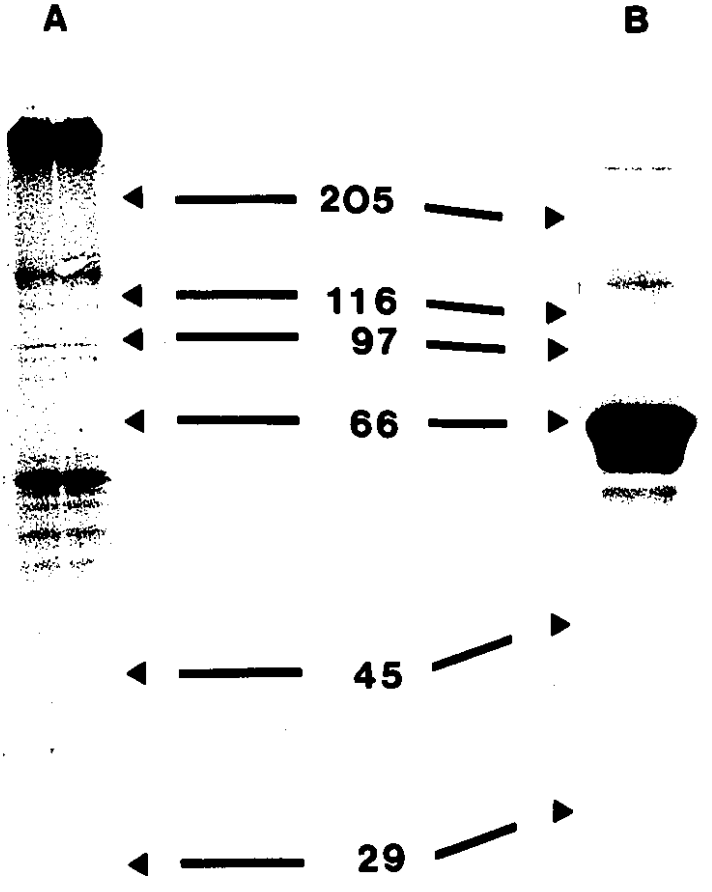
This final rather straightforward purification scheme took some time to develop because of the instability of the enzyme once isolated from the native membrane. As shown before (Rea and Poole, 1986), the solubilized H+-PPase required the presence of detergent, glycerol and phospho-

lipids at all stages of its purification in order to retain its activity.

Initially, when preparing samples for SDS-PAGE, phospholipids and detergent were removed from the TCA precipitated pellets by washing with acetone followed by ethanol, or by using the method of Wessel and Flügge (1984), which involves partitioning the components in a methanol/chloroform/water mixture. After either treatment, the samples were placed in a 95°C bath for 5 minutes before applying them to the gel. These procedures were, however, abandoned when it became evident that the H+-PPase was precipitating and aggregating irreversibly in organic solvents or when heated (Fig. 2.4)

Figure 2.4. **Effect of boiling on electrophoretic migration of the H+-PPase.*** In preparation for SDS/PAGE, aliquots of partially purified H+-PPase from the same column fraction were A) boiled for 5 min in SDS sample buffer or B) incubated overnight at 4°C in SDS sample buffer and electrophoresed. Proteins are Coomassie stained. Arrows indicate molecular weight markers.

* This figure was not included in the published version.


A
B
205
116
97
66
45
29

CHAPTER 3

Radiation-inactivation analysis of vacuolar H^+-ATPase and H^+-pyrophosphatase from *Beta vulgaris* L.

Functional sizes for substrate hydrolysis and for H^+ transport.

3.1. ABSTRACT

The functional sizes of the vacuolar H^+-ATPase (V-ATPase, EC 3.6.1.34) and H^+-pyrophosphatase (PPase, EC 3.6.1.1) from vacuolar membranes of red beet (Beta vulgaris L.) were estimated by radiation inactivation, both for substrate hydrolysis and for H^+ transport. For the V-ATPase, the radiation-inactivation size for H^+ transport was 446 (403 - 497) kDa and that for ATP hydrolysis was 394 (359 - 435). The low values of both these estimates suggest that not all subunits which may co-purify with V-ATPases are required for either hydrolysis or transport. For the PPase, the radiation-inactivation size for hydrolysis was 91 (82 - 103) kDa suggesting that the minimum functional unit for hydrolysis is the 81 kDa monomer. In contrast to the V-ATPase, the PPase gave a radiation-inactivation size for transport which was 3- to 4-fold larger than that for hydrolysis (two estimates for transport gave 307 and 350 kDa), indicating that a single catalytic subunit is insufficient for transport activity.

3.2. INTRODUCTION

Plant vacuoles are acidified by a typical vacuolar-type H^+-ATPase (V-ATPase, EC 3.6.1.34). Vacuolar H^+-ATPases are found in a variety of endomembrane organelles of plants, animals and fungi, and are evolutionarily related to the F_1F_o-ATPases of mitochondria, chloroplasts and eubacteria (Nelson, 1989). The complex subunit composition of these enzymes is now known to a first approximation (Arai et al., 1988; Matsuura-Endo et al., 1990; Parry et al., 1989). In addition, plant vacuoles have a second proton-translocating enzyme, an H^+-pyrophosphatase (PPase, EC 3.6.1.1), which also appears to serve the purpose of acidifying the vacuole (Hedrich et al., 1989; Rea & Sanders, 1987). The hydrolytic subunit of the latter enzyme has recently been purified and its cDNA has been cloned (see DISCUSSION) but much remains to be done to understand its complex kinetics (White et al., 1990) and subunit composition.

An oligomeric structure for the PPase has been suggested by the behaviour of the PPase in non-denaturing gel electrophoresis (Maeshima, 1990), cross-linking experiments (Maeshima, 1990) and the behaviour of the enzyme in gel filtration columns (Sarafian & Poole, 1989). However, these studies did not resolve the question of the minimum functional unit required for either pyrophosphate hudrolysis or for H^+ transport.

Radiation-inactivation studies have been invaluable in

determining the functional size of a variety of enzymes in relation to their polypeptide composition (Kempner & Fleischer, 1989). Several reports have set the radiation-inactivation size of V-ATPases at about 400 - 500 kDa for ATP hydrolysis, although an estimate of molecular size by summation of associated polypeptides gives values of 650 - 750 kDa (see 3.5. DISCUSSION). In the case of the PPase, radiation-inactivation sizes of 160 kDa (130 to 241) (Chanson and Pilet, 1989) and 88±8 kDa (Fraichard et al, 1991) have been reported for pyrophosphate hydrolysis. The enzyme has been identified on SDS/polyacrylamide gels as a polypeptide of approximately 67 kDa (Britten et al., 1989; Maeshima & Yoshida, 1989; Sarafian & Poole, 1989), however, the cDNA-deduced size indicates that the correct estimate for the hydrolytic subunit is 81 kDa (Sarafian et al., 1992a). It remains therefore to determine whether the minimal functional unit for hydrolysis is a monomer or a dimer.

The radiation-inactivation size for H^+ transport as opposed to hydrolysis has not previously been investigated for either the V-ATPase or the H^+-PPase. The present study provides additional information on the radiation-inactivation of these two enzymes, including estimates of functional size both for substrate hydrolysis and for H^+ transport.

3.3. MATERIAL & METHODS

3.3.1. Tonoplast preparation.

Vacuolar membranes (tonoplast) were prepared from fresh red beet (Beta vulgaris L.) roots as described by Rea & Poole, 1985, Sarafian & Poole, 1989. The membranes were suspended in a medium containing 10% w/v glycerol, 1 mM EDTA, 5 mM dithiothreitol, 2.45 mM BTP and 2.55 mM Mes adjusted to pH 8.0 at 4° C and stored at -70°C.

3.3.2. Radiation inactivation.

Tonoplast fractions were purged with nitrogen and irradiated in dry ice (-78°C) with γ rays in a Gammacell 220 ^{60}Co irradiator for various lengths of time according to the method of Beauregard et al. (1987). Non-irradiated controls were treated in the same way except for the irradiation. The relationship between radiation dose (Mrad) and molecular size was established using the equation:

$$\log \text{molecular size} = 5.89 - \log D_{37,t} - 0.0028t \qquad (I)$$

where D_{37} is the dose required to decrease enzyme activity to 37% of initial rate and t is the temperature (°C) at which the irradiation was applied (Beauregard et al., 1987).

3.3.3. Enzyme assays and data analysis.

Glucose 6-phosphate dehydrogenase activity was measured

as described (McIntyre & Churchill, 1985).

Phosphohydrolase activity of the PPase was estimated at 37 °C for 10 minutes by the method of Ames (1966) in 0.3 ml reaction volumes containing 14.7 mM BTP and 15.3 mM Mes to give pH 8.0, 100 mM KCl, 5 μM Gramicidin D, 0.6 mM K-PP_i and 0.5 mM $MgCl_2$. ATPase assays were performed under similar conditions except for the substrate which was 1.5 mM ATP and 3 mM $MgCl_2$. Hydrolytic activities of the unirradiated controls (μmole of substrate hydrolysed/h per mg) were 11 - 13 for the ATPase and 11 - 14 for the PPase.

H^+-transport activity was measured as the initial rate of quenching of fluorescence by the dye Acridine Orange (Bennett & Spanswick, 1983) at room temperature (22 °C) in a Perkin-Elmer Fluorescence Spectrophotometer at excitation and emission wavelengths of 495 and 540 nm respectively. Control and irradiated samples containing 30-50 μg protein (constant within each experiment) were transferred to 1 ml of reaction medium containing 2.45 mM BTP and 2.55 mM Mes to give pH 8.0, 50 mM KCl, 5 μM Acridine Orange and either 0.3 mM PP_i or 1.5 mM ATP. The reaction was started by the addition of 3 mM $MgSO_4$.

In order to minimize post-irradiation damage, assays for H^+ transport were performed immediately after thawing of the samples (each sample being allowed to thaw separately immediately before assay) and assays for hydrolytic activity were done by adding the assay medium directly to the frozen samples.

For each experimental condition, two to three different membrane preparations were irradiated and assayed at different occasions, with similar results. Each data point for hydrolysis or transport represents the mean result from the irradiation and assay of three independent membrane samples. Regression lines were fitted with the least squares method. Molecular size estimates given are calculated from D_{37} ±S.E.M. values.

3.3.4. SDS/PAGE

One-dimensional SDS gel electrophoresis was performed in a Bio-Rad Protean II Slab Cell according to Laemmli (1970). The irradiated samples were treated with SDS sample buffer, incubated at 4 °C overnight and heated to 60 °C for 15 minutes immediately prior to loading on 10% acrylamide gels. (Sarafian & Poole, 1989). Prestained markers (Bio-Rad) were run alongside the samples.

3.3.5. Western Blot and Immunodetection.

The SDS/polyacrylamide gel was blotted on to nitrocellulose (Millipore HA) in a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) equipped with card electrodes. The transfer was done in Towbin's buffer (Towbin et al., 1979) at 0.1 A overnight followed by 1 h at 0.7 A. The blot was probed with anti-PPase antibody (1:300 dilution) followed by an incubation in 1μCi 125I-Protein A. The blot was air dried and exposed to X-ray film. The autoradiogram was used as a guide

to locate and cut the immunoreacted bands from the blot. Radioactivity was measured by scintillation counting.

3.3.6. Antibodies.

Polyclonal antibodies raised against mung-bean hypocotyl vacuolar PPase were kindly given to us by Dr. M. Maeshima (Hokkaido University, Japan). The antibodies specifically recognized the 67 kDa PPase polypeptide in red beet root vacuolar membranes.

3.3.7. Protein assay.

Membrane protein was measured by a modified Lowry method (Bennett, 1982).

3.4. RESULTS

3.4.1. Radiation inactivation of glucose 6-phosphate dehydrogenase.

This enzyme has been widely used as an internal marker for radiation-inactivation studies. Its molecular mass, estimated by a variety of methods, is 104 kDa (McIntyre & Churchill, 1985). Glucose 6-phosphate dehydrogenase at 10 μg per ml was added to vacuolar membranes and, after exposure to various doses of radiation, the size of the enzyme was calculated from triplicate samples using equation (I). The deduced molecular size was 93±2 kDa. Molecular size calculations (below) for the PPase and ATPase have not been corrected for

the underestimation of the standard.

3.4.2. Radiation-inactivation of the V-ATPase.

When tonoplast vesicles were exposed to increasing doses of ionizing radiation, both hydrolytic and H+ pumping activities of the vacuolar ATPase decreased in an exponential manner (Fig. 3.1). However, at relatively low radiation doses, an initial non-linear component was observed in the log-transformed inactivation curve for transport. A more detailed discussion on the possible reasons for this phenomenon is provided later, but for determination of D_{37} values, only those points showing a linear decline in activity were used (open symbols in Fig. 3.1). The regression line intercept with the ordinate was determined and used as the basis for calculating 37% of activity. For the V-ATPase, size estimates from the assay of hydrolytic activity in native membranes and from measurements of H^+-pumping were 394 kDa (359 - 435 kDa) and 446 kDa (403 - 497 kDa) respectively (Fig. 3.1).

3.4.3. Radiation inactivation of H^+-PPase.

As in the case of the V-ATPase, the vacuolar PPase showed an exponential decrease in both hydrolytic and transport activity with increasing dose of radiation over a certain minimum dose (Fig. 3.2). Molecular size determinations were made on the linear component, excluding the low dose values (closed symbols).

Figure 3.1. **Inactivation of the vacuolar ATPase by γ radiation.** Shown is the ATPase H^+-pumping activity (top panel) and hydrolytic activity (bottom panel), in red beet tonoplast after exposure to various doses of γ radiation at -78°C. Results are expressed as the logs of the percentage activity remaining after irradiation. Regression lines were fitted by the least squares method. Symbols (○, □, △) represent separate membrane preparations with each point averaged from simultaneous triplicate irradiations. Closed symbols indicate data not used in the computation of the regression. Estimated D_{37} values (means±S.E.M.) were 2.88±0.30 (n = 12; r=-0.99) and 3.26±0.31 (n=20; r=-0.97) for H+ transport and ATP hydrolysis respectively, giving molecular sizes of 446 (403-497) kDa and 394 (359 - 435) kDa, respectively.

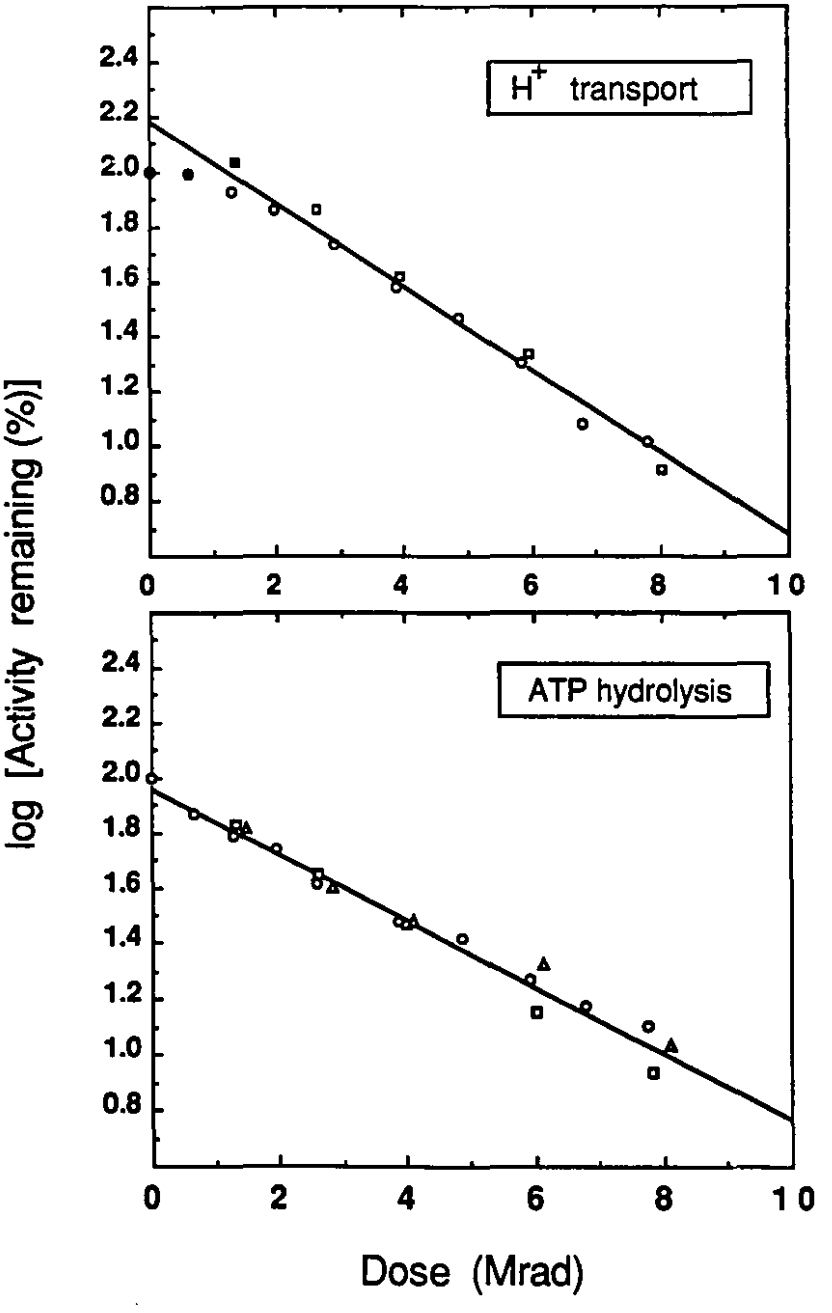
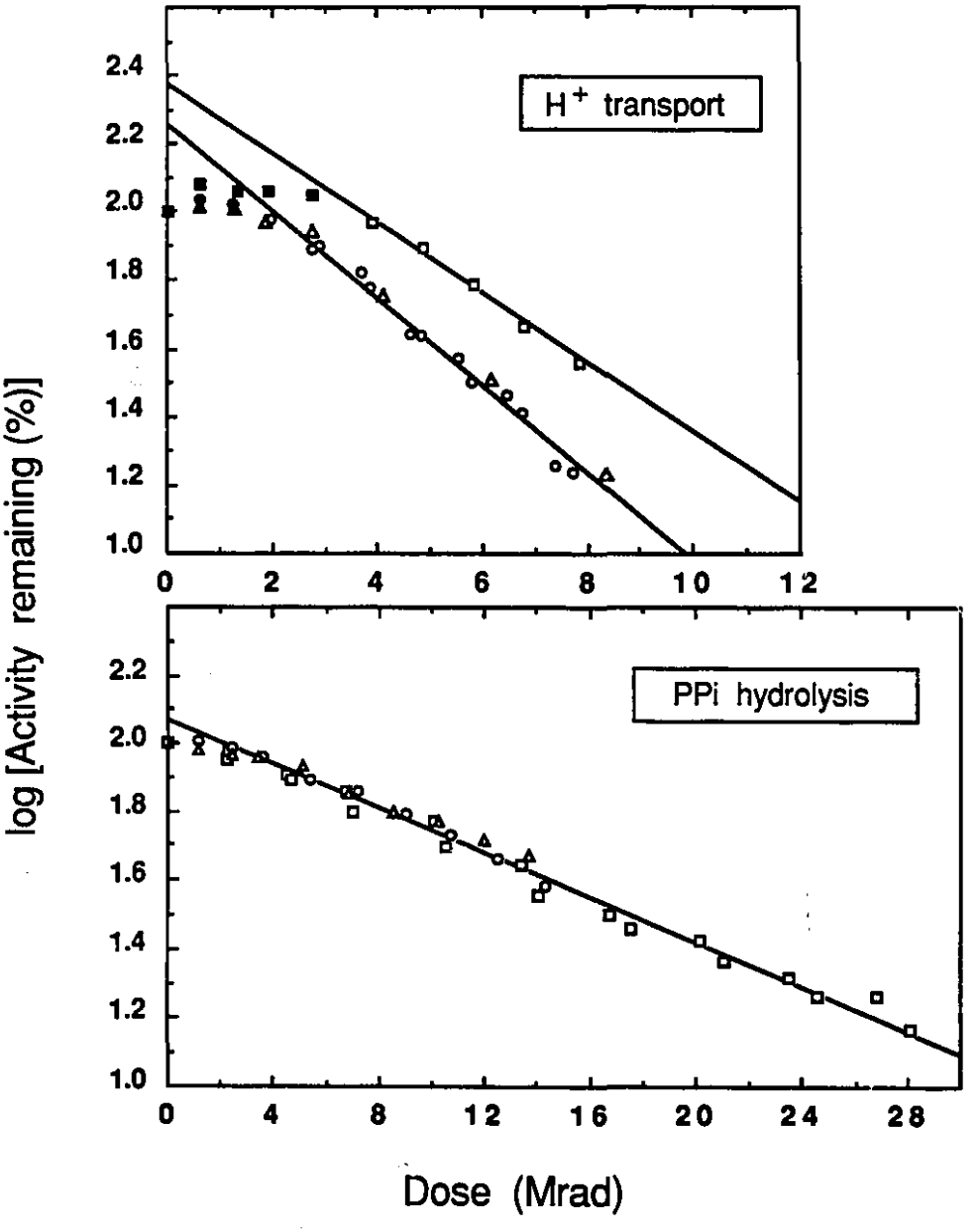

H+ transport
ATP hydrolysis
log [Activity remaining (%)]
Dose (Mrad)
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
2
4
6
8
10

Figure 3.2. **Inactivation of the vacuolar PPase by γ radiation.** Shown are the pyrophosphatase H^+-pumping activity (top panel) and hydrolytic activity (bottom panel), in red beet tonoplast (note difference in scale of the abscissae). Activities are expressed as the logs of the percentage activity of remaining activity after irradiation. The two sets of values obtained for H^+-transport differ mainly as a result of a different activation pattern at low doses in different batch of membranes. Regression lines were fitted by the least squares method. Closed symbols indicate data not used in the calculation of the regressions. Symbols (○, □, △) represent separate membrane preparations with each point averaged from simultaneous triplicate irradiations. Estimated D_{37} values (means±S.E.M.) were 4.18±0.25 (n=5; r=-0.99) for the upper H^+ transport curve and 3.46±0.20 (n=18; r=-0.99) for the lower H^+ transport curve with corresponding molecular sizes of 307 (289 - 326) kDa and 350 (333 - 371) kDa. The D_{37}±S.E.M. for PPi hydrolysis was 14.05±1.65 (n=37; r=-0.99) which yielded a molecular size of 91 (82 - 103) kDa.


H+ transport
PPi hydrolysis
log [Activity remaining (%)]
Dose (Mrad)
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
2
4
6
8
10
12
16
20
24
28

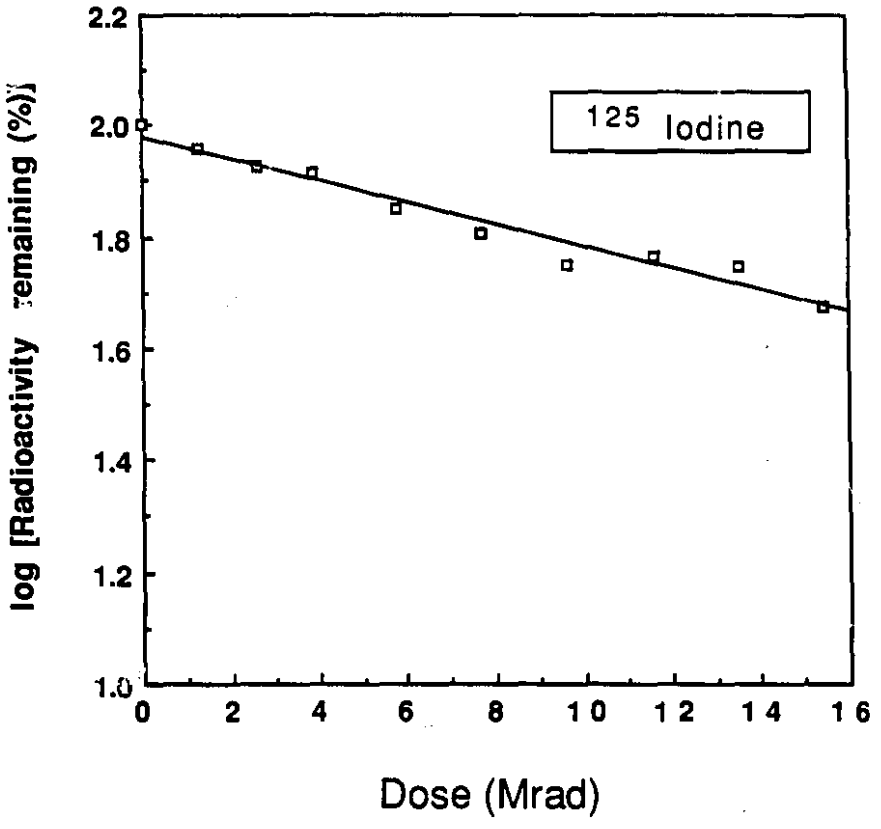
The radiation-inactivation size of PPase for hydrolysis was 91 kDa (82 - 103 kDa), which would correspond to a monomer of the 81 kDa subunit predicted from the cDNA sequence.

In the case of transport assays on this enzyme, it became apparent that the different sets of values obtained using different batches of membranes could not be grouped into a single regression line. Even though the slopes of the regression lines through the linear portion of the curves were comparable, large differences in the initial, non-linear part of the curve for one batch of membranes indicated that the factors causing the rise in activity at low doses were variable. The molecular size estimates from the two inactivation curves for H^+-transport activity (Fig. 3.2) are 307 kDa (289 - 326 kDa) and 350 kDa (333 - 371 kDa) for the top and bottom curves respectively. Despite the difference between these two estimates, it is clear that the radiation-inactivation size for transport is 3- to 4-fold larger than that for substrate hydrolysis.

3.4.4. Target size for destruction of the PPase catalytic subunit.

Irradiated tonoplast vesicles were solubilized in SDS and subjected to gel electrophoresis. The proteins were blotted on to nitrocellulose and probed with antibodies against purified PPase. Antibody binding was quantified by the use of ^{125}I-Protein A. A single band running at 67 kDa,

Figure 3.3. **Immunodetection of irradiated PPase by ^{125}I-Protein A.** Tonoplast vesicles were irradiated and subjected to SDS/PAGE. Proteins were transferred to nitrocellulose and incubated with antibody to PPase, followed by 1 μCi of ^{125}I-Protein A. The radioactivity associated with the PPase was measured in a scintillation counter. Results are expressed as the log of the radioactivity as a percentage of that in the non-irradiated control. The regression line was fitted by the least squares method. The D_{37}±S.E.M. value was 20.1±1.3 (n=10; r=-0.98). The corresponding target size for the immunodetected polypeptide was 64±4 kDa.


log [Radioactivity remaining (%)]
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
2
4
6
8
10
12
14
16
125 Iodine
Dose (Mrad)

corresponding to the catalytic subunit, was observed. Figure 3.3 shows a regression curve deduced from the radioactivity bound to the PPase after exposure to increasing radiation doses. The target molecular size deduced from quantification of the polypeptide was 64±4 kDa.

These results suggest that radiation destroys only one subunit with each ionizing hit. Although this result is based on a small sample, it serves to confirm that radiation destroys only one 81 kDa subunit with each ionizing hit, i.e., the energy of ionization is not transferred from one polypeptide to another.

The discrepancy between the estimated target size and the size predicted by cDNA cloning is perhaps due to incomplete electrophoretic transfer to nitrocellulose. Several Western blots of irradiated samples showed that lower concentrations of polypeptide transfer more completely than higher ones, resulting in a lower estimate of molecular size. Judging from the small amount of protein remaining in the gels, however, this effect would not be large enough to invalidate the above conclusion.

3.5. DISCUSSION

3.5.1. Reliability of transport measurements.

Measurements of transport across membranes are affected by the size and passive conductance of the vesicles. Previous studies on the glucose and phosphate transporters in brush

border membrane vesicles showed that radiation doses of up to 8.5 Mrad did not significantly alter vesicle size and integrity (Beliveau et al., 1988). Since the effect of radiation beyond this level is not known, samples treated with doses higher than 8 Mrads were not used in the analysis of transport data. In addition, two aspects of the present results suggest that there were no important changes in the size or conductance of the vesicles over the course of the radiation treatments. First, after the initial anomalies at low doses of radiation (see below), the measured transport activities of both enzymes showed the expected simple exponential decay. Secondly, for the V-ATPase, the radiation-inactivation size determined from transport measurements was only slightly different from that determined from hydrolytic activity.

Proton transport activity was measured as the initial rate of quenching of the dye Acridine Orange. A theoretical justification of this widely-used method is given by Bennett & Spanswick (1983). When the experimental conditions are properly chosen, the initial rate of change of either fluorescence or absorbance of the dye can give a good measure of the relative rates of proton transport. For example, in a study of vacuolar H^+-ATPase, Palmgren & Sommarin (1989) found that the initial response of the dye closely paralleled the rate of ATP hydrolysis over a wide range of enzyme activity. In the present work, it is not possible to say whether the difference in radiation-inactivation size for transport (446 kDa) versus hydrolysis (394 kDa) by the ATPase reflects a

real property of the enzyme, or a non-ideal behaviour of either the acridine orange fluorescence or the vesicle permeabilities. In any case, the relatively close agreement between the radiation-inactivation sizes for transport and hydrolysis by the V-ATPase serves as an internal control for the more interesting case of the PPase, in which the two estimates are markedly (3- to 4-fold) different.

3.5.2. Anomalies of the inactivation curves.

Deviations from simple exponential decay of activity were seen at low radiation doses, for transport activities of the V-ATPase and of the PPase (Figs. 3.1 & 3.2). Such deviations, which represent an increase in activity of those enzyme molecules not 'hit' by irradiation, are more commonly found with membrane enzymes than with soluble ones (Kincaid et al., 1981). The deviations have been interpreted either in terms of the inactivation of a large-molecular weight inhibitor (Harmon et al., 1980), or in terms of the dissociation of oligomers of the enzyme into monomers with increased activity (Kincaid et al., 1981). In the present case, this would mean dissociation of aggregates of the holoenzyme into single functional units of about 400 kDa each (for the V-ATPase) or about 320 kDa each (for the PPase).

It is important to consider whether the anomalies seen at low radiation doses in any way invalidate estimates of molecular size from the subsequent exponential decay. When such anomalous effects cannot be clearly distinguished, they

can lead to erroneous values of radiation-inactivation size (Potier & Giroux, 1985; Simon et al., 1982). In the present case, the anomalies are limited to low doses, and are followed by simple exponential decay curves. In this case, regardless of the interpretation of the initial activation, the final slopes and corresponding radiation-inactivation sizes should not be affected (Harmon et al., 1980; Verkman et al., 1984). Nevertheless, it seems possible that these initial activation effects are responsible for the slight difference in final slope between the two curves for H^+ transport of the PPase in Fig. 3.2.

3.5.3. Radiation-inactivation size of the V-ATPase.

The molecular sizes calculated from Figs. 3.1, 3.2 and 3.3 for the V-ATPase and the PPase under various conditions are collated in Table 3.1. For the V-ATPase, the estimates for hydrolysis and for transport are in the same range, around 400 - 450 kDa. These estimates are in agreement with previously reported radiation-inactivation sizes for this enzyme. In corn coleoptiles, values of 387 ±19 kDa (Wang et al., 1989) and 386 kDa (309 - 520 kDa) (Mandala & Taiz, 1985) have been reported, as well as an estimate of 405 ±25 kDa from mung-bean hypocotyls (Wang et al., 1989) and a slightly higher value of 542 kDa (374 - 642 kDa) from maize roots (Chanson & Pilet, 1989). In fungi, a size of 410 - 530 kDa has been reported for yeast (Hirata et al., 1989) and 520 kDa for Neurospora (Bowman et al., 1986).

The similarity in inactivation sizes for ATPase hydrolytic and pumping activities suggests that the same minimal functional size is required for both functions. This conclusion was also reached by Anraku and his colleagues (Hirata et al., 1989; Uchida et al., 1988), based on the observation that hydrolysis under normal conditions, either in native membranes or in the Zwittergent-solubilized enzyme, is sensitive to dicyclohexylcarbodiimide, which blocks the proton channel of V-ATPases (Sun et al., 1987) in the same

Table 3.1. **Summary of results obtained for radiation-inactivation size.** Results are molecular sizes estimated from D_{37} (means±S.E.M.) values using equation (I).

	Molecular mass (kDa)	
	ATPase	PPase
Polypeptide subunit		64±4
		81*
Hydrolysis	394 (359 - 435)	91 (82 - 103)
H^+ transport	446 (403 - 497)	307 (289 - 336)
		350 (333 - 371)

* Estimated from cDNA sequence

manner as for F_1F_o-ATPases. This group also showed (Hirata et al., 1989) that the radiation-inactivation size of yeast V-ATPase for normal hydrolysis was 410 - 530 kDa, corresponding to the above estimates, but that the functional size decreased to 90 - 110 kDa for single-cycle hydrolysis, under which conditions it was insensitive to the carbodiimide. The latter value would correspond to a single heterodimer of the A and B subunits, which are normally present in 3 copies in the catalytic head of the enzyme (Arai et al., 1988).

The radiation-inactivation size of the V-ATPase under normal conditions appears, however, to underestimate the proposed size (759 kDa) arrived at by addition of the associated subunits in their stoichiometric ratios in clathrin coated vesicles (Arai et al., 1988). Addition of V-ATPase-associated subunits in plants, using the same plausible stoichiometry, gives total sizes of 709 kDa (based on Parry et al., 1989) or 647 kDa (based on Matsuura-Endo et al., 1990). In the case of mitochondrial ATPase, the radiation-inactivation size (460 kDa) more closely approximates the value (530 kDa) obtained by addition of subunits (Bowman et al., 1986). The discrepancy between estimates of size of the V-ATPase obtained with the two methods remains to be elucidated. It may be that not all of the subunits found to be associated with the purified enzyme are required for either hydrolysis or transport. In particular, the radiation-inactivation data suggest that a large subunit of 100 - 115 kDa sometimes associated with purified preparations of V-

ATPase in both plants (Parry et al., 1989) and animals (Nelson, 1989) is unlikely to be required for either ATP hydrolysis or for H^+ transport. There is, however, evidence that a homologous polypeptide in yeast is required for V-ATPase assembly (M. F. Manolson, personal communication).

3.5.4. Radiation-inactivation size of the H^+-PPase.

The radiation-inactivation size for PPase activity (Table 3.1) was found to be 91 kDa (82 - 103 kDa), in agreement with a recently reported value of 88±8 kDa (Fraichard et al., 1991) for PPase in Acer vacuolar membranes rather than a previously reported estimate of 130 - 241 kDa (Chanson & Pilet, 1989). The size of the polypeptide has been determined from the cDNA sequence to be 81 kDa (Sarafian et al., 1992b), somewhat larger than the SDS/PAGE estimates of about 67 kDa for the purified hydrolytic polypeptide (Britten et al., 1989; Maeshima & Yoshida, 1989; Sarafian & Poole, 1989).

Purified preparations of vacuolar PPase contain a single polypeptide of about 67 kDa (Britten et al., 1989; Maeshima & Yoshida, 1989). Antibodies to this polypeptide inhibit PPase activity (Maeshima & Yoshida, 1989). Moreover, labeling of the polypeptide and inhibition of enzyme activity by N-ethylmaleimide are both protectable by $MgPP_i$ (Britten et al., 1989). Since purified preparations are active in hydrolysis, and no other polypeptides co-purify with enzyme activity, it is concluded that no other polypeptides are required for pyrophosphate hydrolysis. The functional unit is therefore a

monomer of the 81 kDa polypeptide.

In contrast to the above results for pyrophosphate hydrolysis, the radiation-inactivation size for H^+ transport by the PPase was found in two different preparations to be 307 kDa (289 - 326 kDa) or 350 kDa (333 - 371 kDa). The minimum functional size for transport is therefore 3- to 4-fold larger than for hydrolysis. Recent evidence suggests that purified preparations of the 81 kDa polypeptide can be reconstituted in a form competent in H^+ transport (C. Britten and P.A. Rea, personal communication), which would suggest that the functional unit for transport activity probably consists of several identical subunits of the 81 kDa polypeptide.

CHAPTER 4

Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump (H^+-PPase) of Arabidopsis thaliana

4.1. ABSTRACT

The energy-dependent transport of solutes across the vacuolar membrane (tonoplast) of plant cells is driven by two H^+ pumps: a vacuolar ("V-type") H^+-ATPase and a H^+-translocating (pyrophosphate-energized) pyrophosphatase (H^+-PPase). The H^+-PPase, like the V-type H^+-ATPase, is abundant and ubiquitous in the vacuolar membranes of plant cells and both enzymes make a substantial contribution to the H^+-electrochemical potential difference across the tonoplast. Here, we report the cloning and sequence of cDNAs encoding the tonoplast H^+-PPase of Arabidopsis thaliana. The protein predicted from the nucleotide sequence of the cDNAs is constituted of 770 amino acids and has a molecular mass of 80,800 Da. Immunological and direct sequence data confirm the identity and deduced amino acid sequence of the clones. The polypeptide encoded by the cDNAs is an unusually hydrophobic, integral membrane protein and the structure derived from hydropathy plots contains at least 13 transmembrane spans. Since the tonoplast H^+-PPase appears to be constituted of one type of polypeptide and genomic Southern analyses indicate that the gene encoding the 80,800 Da polypeptide is present in only a single copy in the genome of Arabidopsis, it is suggested that the H^+-PPase has been cloned in its entirety. The lack of sequence identities between the tonoplast H^+-PPase and any other characterized H^+ pump or PPi-dependent enzyme imply unique evolutionary origins for this enzyme and membership of a completely new class of ion translocase.

4.2. INTRODUCTION

General acceptance of the chemiosmotic hypothesis (Mitchell, 1961) has given rise to the view that membrane-bound H^+-pumps constitute the primary transducers by means of which living cells interconvert light, chemical and electrical energy. Through the establishment and maintenance of transmembrane electrochemical gradients, H^+ pumps energize the transport of other solutes or, in the special case of the energy-coupling membranes of mitochondria, chloroplasts and bacteria, transduce the H^+-electrochemical gradient generated by membrane-linked redox reactions to the synthesis of ATP (Mitchell, 1961). Given the multitude of biological reactions energized by ATP, primary H^+-translocation and the interconversions of ATP have come to be recognized as the principal generators of usable energy in the cell. Against this background it is intriguing that the vacuolar membrane (tonoplast) of plant cells contains not only a H^+-ATPase (EC 3.6.1.3) (Sze, 1985; Nelson & Taiz, 1989) but also an inorganic pyrophosphate-energized H^+ pump (H^+-PPase; EC 3.6.1.1) (Rea & Sanders, 1987). Both enzymes catalyze inward electrogenic H^+-translocation (from cytosol to vacuole lumen) but the H^+-PPase has the unusual characteristic of exclusively utilizing PPi as energy source (Rea & Poole, 1986).

While the universality of ATP as cellular energy cur-

rency and its primacy in the energization of primary ion translocation is not in doubt - cation-translocating ATPases capable of transporting H^+, Na^+, K^+ and/or Ca^{2+} have been found in essentially all cells - the existence of a PPi-driven vacuolar H^+ pump in plant cells challenges conventional notions of the significance of PPi for cell metabolism. The undergraduate textbook account of PPi, originally formulated by Kornberg (1963), is that it is simply a nucleotidyl transfer coproduct whose hydrolysis by soluble PPases serves to pull the primary (usually biosynthetic) reaction to completion (Mathews & van Holde, 1990). It is now clear, however, that such a scheme is deficient on three counts. First, it is not a necessary condition of the Kornberg scheme that the energy liberated during PPi hydrolysis be simply lost as heat. The same driving force for biosyntheses would, for example, be provided by a H^+-translocating PPase which lowers cellular PPi concentration while conserving a proportion of the energy of the phosphoanhydride bond in the form of a transmembrane H^+ gradient. Second, numerous energy-conserving PPi-dependent enzymes, in addition to the tonoplast H^+-PPase, have been identified - notable examples are the H^+-translocating PPases of phototrophic bacteria (Baltscheffsky & Nyrén, 1987; Nyrén et al., 1991) and mitochondria (Lundin et al., 1991) and the PPi-dependent phosphofructokinases of some protozoa (Reeves, 1976), certain bacteria (O'Brien et al., 1975) and many algae and plants (ap Rees, 1988). Third, recent investigations have shown that when cytosolic PPi

levels are actually measured, they are almost invariably higher than would be expected from the exclusive operation of the Kornberg scheme (Weiner et al., 1987; Takeshige & Tazawa, 1989) and commensurate with operation of the tonoplast H^+-PPase at full capacity in vivo (Rea & Sanders, 1987).

Phenomenologically, the tonoplast H^+-PPase appears to be important for plant cell function: it is widespread, active and abundant. The enzyme is ubiquitous in the vacuolar membranes of plant cells (Rea & Sanders, 1987) and capable of establishing a H^+ gradient of similar, and often greater, magnitude than the H^+-ATPase on the same membrane (Wang et al., 1986; Maeshima & Yoshida, 1989). The 64.5-73 kDa, substrate- (MgPPi-) binding subunit of the H^+-PPase constitutes between 1% (Britten et al., 1989) and 10% (Maeshima & Yoshida, 1989) of total vacuolar membrane protein and the purified enzyme has a turnover number of between 50 and 100 s^{-1}, depending on the source and preparation (Britten et al., 1989; Rea et al., 1992a). Thus it is evident, when account is taken of the fact that the vacuole of the mature plant cell can constitute 90% of total intracellular volume, that the potential bioenergetic impact of the tonoplast H^+-PPase is great.

In the light of these considerations and the unique status of PPi as the limiting case of a high-energy phosphate, insight into the evolutionary origins and structure of this novel H^+-translocase should be of importance not only for understanding vacuolar energization and cellular PPi metabolism in plants, in particular, but also for elucidating

the mechanisms underlying biological energy transduction, in general. Towards this end, we have sought to isolate the gene(s) encoding the tonoplast H^+-PPase for detailed structural and molecular genetic analyses.

The present paper describes the molecular cloning of cDNAs encoding the tonoplast H^+-PPase of Arabidopsis thaliana. While the predicted structural characteristics of the protein encoded by the cDNAs are consistent with the enzyme's capacity for PPi-dependent transtonoplast H^+ pumping and align with direct sequence data obtained from purified H^+-PPase poly- peptide, the deduced amino acid sequence of the polypeptide encoded by the clones does not show any identities with any other sequenced ion pump. It is therefore concluded that the tonoplast H^+-PPase is representative of a completely new class of ion translocase.

4.3. MATERIALS AND METHODS

4.3.1. Materials

The plant materials were Arabidopsis thaliana cv. Columbia, Vigna radiata cv. Berken and Beta vulgaris L. cv. Detroit Dark. Rabbit polyclonal antibody raised against tonoplast H^+-PPase purified from etiolated hypocotyls of mung bean (Vigna radiata) was a kind gift from Dr. M. Maeshima to V.S. and R.J.P. The Arabidopsis cDNA libraries constructed in λZAP and λgt10, respectively, were provided by Drs. Joe Ecker and Nigel Crawford.

4.3.2. Preparation of Membranes

Tonoplast vesicles were isolated from red beet (Beta vulgaris L.) storage root and etiolated hypocotyls of mung bean (Vigna radiata) as described previously (Rea & Poole, 1985; Sarafian & Poole, 1989). Microsomes were prepared from 10 day-old, dark-grown seedlings of Arabidopsis thaliana by homogenization and differential centrifugation. Seedlings (12 g fresh weight) were homogenized with a mortar and pestle in 100 ml extraction buffer [1.1 M glycerol/5 mM Tris-EDTA/5 mM DTT/1% PVP-40/1 mM PMSF/70 mM Tris-Mes (pH 8.0)] and the homogenate was centrifuged at 6,000 x g for 20 min. The pellet was discarded and the supernatant was centrifuged at 100,000 x g for 30 min. The microsomal pellet was resuspended in 1-2 ml suspension medium [1.1 M glycerol/1 mM Tris-EGTA/5 mM DTT/30 mM Tris-Mes (pH 8.0)], frozen in liquid nitrogen and stored at -85°C.

4.3.3. Preparation of Antibody for Immunoscreens

The rabbit polyclonal antibody used in these studies had been raised against total peak tonoplast H^+-PPase chromatographic fractions purified from etiolated hypocotyls of Vigna (Maeshima & Yoshida, 1989). To ensure monospecificity, the antibody was affinity-purified against the 66 kDa substrate- (MgPPi-) binding subunit of the H^+-PPase from Vigna by the method of Sambrook et al (1989). Vigna tonoplast protein (1 mg) was subjected to preparative SDS/PAGE on a single-well, 1.5 mm 7-12% concave exponential gradient gel

(O'Farrell, 1975) and electrotransferred to nitrocellulose (Towbin et al., 1979). The nitrocellulose filters were rinsed for 10 min in TTSB and blocked for 1 h in TTSB containing 5% (wt/vol) powdered skimmed milk. After 3 x 5 min rinses in TTSB, the filters were incubated overnight in antibody (1/300 dilution). Two narrow vertical strips were cut from each end of the filters and incubated in TTSB containing HRP-conjugated sheep antirabbit IgG (Boehringer Mannheim) for 1 h. Immunoreactive bands were visualized with H_2O_2/diaminobenzidine/$NiCl_2$ (Harlowe & Lane, 1988). The immunoreacted strips were aligned with the remainder of the filters and a narrow horizontal band corresponding to the 66 kDa subunit of the H^+-PPase was excised. The excised strip was rinsed in 0.15 M NaCl for 20 min, rinsed with TSB and bound antibody was eluted with 0.2 M glycine/1 mM EGTA (pH 2.8). The eluate was immediately neutralized with Tris-Base and made 1 X with TSB and 0.02% with sodium azide. Affinity-purified antibody was used after 100-fold dilution with TSB.

4.3.4. Polypeptide Sequencing

The H^+-PPase was purified from isolated Beta tonoplast vesicles by the methods of Sarafian and Poole (1989) and Britten et al.,(1989) respectively, and aliquots (20-50 μg) of the purified enzyme were separated by SDS/PAGE. The gels were polymerized for at least 18 h before use, high purity chemicals were used throughout and thioglycollate (0.1 mM) was included in the running buffer to minimize oxidation of

the samples during electrophoresis (Matsudaira, 1988).

Electrophoresed PPase was electrotransferred to nitrocellulose filters and protein was reversibly stained with Ponceau-S (Aebersold et al., 1987). The protein band corresponding to the substrate-binding subunit of the H^+-PPase was excised immediately, destained and shipped on dry-ice to the Harvard Microchemistry Facility, Cambridge, MA, U.S.A., for in situ tryptic digestion and gas-phase sequence analysis by the method of Aebersold et al. (1987).

4.3.5. Immunoscreens of Arabidopsis λZAP cDNA Library

The Arabidopsis cDNA expression library in λZAP was constructed from poly $(A)^+$ RNA isolated from hypocotyls of 3 day-old, dark-grown seedlings (Ecker, J., unpublished). The primary screens were performed by standard procedures (Sambrook et al., 1989) using unfractionated antibody raised against the H^+-PPase purified from Vigna (Maeshima & Yoshida, 1989). The nitrocellulose filters from the plaque lifts were incubated in 0.1% SDS for 1 h followed by 3 x 5 min washes in TTSB. Immunoreaction and immunodetection were as described for the affinity-purification of antibody except that 4-chloro-1-naphthol, instead of diaminobenzidine, was used as chromogen (Harlowe & Lane, 1988). The putative positive plaques were rescreened 2-3 times with affinity-purified antibody to ensure clonal purity. Two of the largest clones containing inserts of approximately 2.1 kb (AVP-1 and AVP-2; Arabidopsis Vacuolar Pyrophosphatase) were analyzed further.

The cDNA insert of pAVP-1 was sequenced completely, after *in vivo* excision, and used for subsequent hybridization screens; the cDNA insert of pAVP-2 was employed to extend the sequence range.

4.3.6. Hybridization Screens of Arabidopsis λgt10 Library with AVP-1

To obtain larger cDNA inserts, an *Arabidopsis* cDNA library constructed in λgt10 was screened using random-primed AVP-1 (Feinberg & Volgentein, 1983). The plaque lifts were prehybridized at 42°C in 50% formamide/5 X SSPE (0.74 M NaCl, 57.5 mM NaH_2PO_4, 6.3 mM EDTA, pH 7.4)/1 X Denhardt's solution/0.1% SDS containing 100 μg/ml denatured, sonicated salmon sperm DNA for 2 h. The plaque hybridizations were performed in the same solution containing random primed cDNA insert at 42°C for 12-16 h after which time the filters were washed twice in 2 X SSC [0.3 M NaCl, 30 mM sodium citrate (pH 7.0)]/0.2% SDS and once in 1 X SSC/0.1% SDS at 65°C for 30 min. The positive clone containing the longest insert was purified and subcloned into the EcoRI site of pBluescript to generate pAVP-3.

4.3.7. Genomic Blot Hybridizations

Genomic DNA was extracted from 14 day-old *Arabidopsis* seedlings (Ausubel *et al.*, 1987). Samples (2 μg) of the DNA were digested with restriction enzyme, electrophoresed on 0.8% agarose gels and transferred to nylon (Nytran45, Schlei-

cher and Schuell) membrane filters. The filters were prehybridized with 6 X SSPE [0.89 M NaCl, 69 mM NaH_2PO_4, 7.6 mM EDTA (pH 7.4)]/10 X Denhardt's solution/0.5% SDS] containing 100 μg/ml denatured, sonicated salmon sperm DNA at 42°C for 2 h. The filter hybridizations were performed in 50% formamide/6 X SSPE/0.5% SDS containing 50 μg/ml denatured, sonicated salmon sperm DNA and random primed cDNA probe (AVP-1, specific activity 1 x 10^8 cpm/μg) at 42°C for 12 h. The hybridized filters were washed twice in 6 X SSPE/0.1% SDS for 30 min at room temperature, twice in 1 X SSPE/0.1% SDS for 20 min at 37°C and once in 0.1 X SSPE/0.1% SDS for 30 min at 65°C. The filters were exposed to Kodak X-Omat AR X-ray film with intensifying screens for 12-16 h at -85°C.

4.3.8. Northern Blot Hybridizations

Total RNA was extracted from 7 day-old, dark-grown Arabidopsis seedlings by the phenol-SDS method (Ausubel et al., 1987) and poly $(A)^+$ RNA was isolated by oligo(dT)-cellulose chromatography. The poly $(A)^+$ RNA was size-fractionated by electrophoresis on 1% agarose gels containing 0.63 M formaldehyde and blotted onto nylon (Nytran-45) membrane filters. The filters were prehybridized as described for the λgt10 screens and hybridized in the same solution containing random-primed AVP-1 cDNA as probe (specific activity 1 x 10^8 cpm/μg) for 15-20 h at 42°C. The filters were washed and exposed to X-ray film as described above for the genomic blot hybridizations.

4.3.9. DNA Sequencing

DNA was sequenced by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using a Sequenase II kit (United States Biochemical). A series of nested deletions derived from the cDNA insert of pAVP-1 were obtained using a double-stranded nested deletion kit (Pharmacia). The remaining sequencing reactions were performed using custom-synthesized oligonucleotide primers.

4.3.10. SDS/PAGE

One-dimensional SDS/PAGE was performed on concave exponential gradient gels. Lipid was removed from the membranes before the addition of denaturation buffer by extraction with acetone:ethanol [1:1 (vol/vol); -20°C) (Parry et al., 1989). The gels were double-stained with silver stain (Bio-Rad) and Coomassie blue by the method of Dzandu et al (1984).

4.3.11. Immunoblots

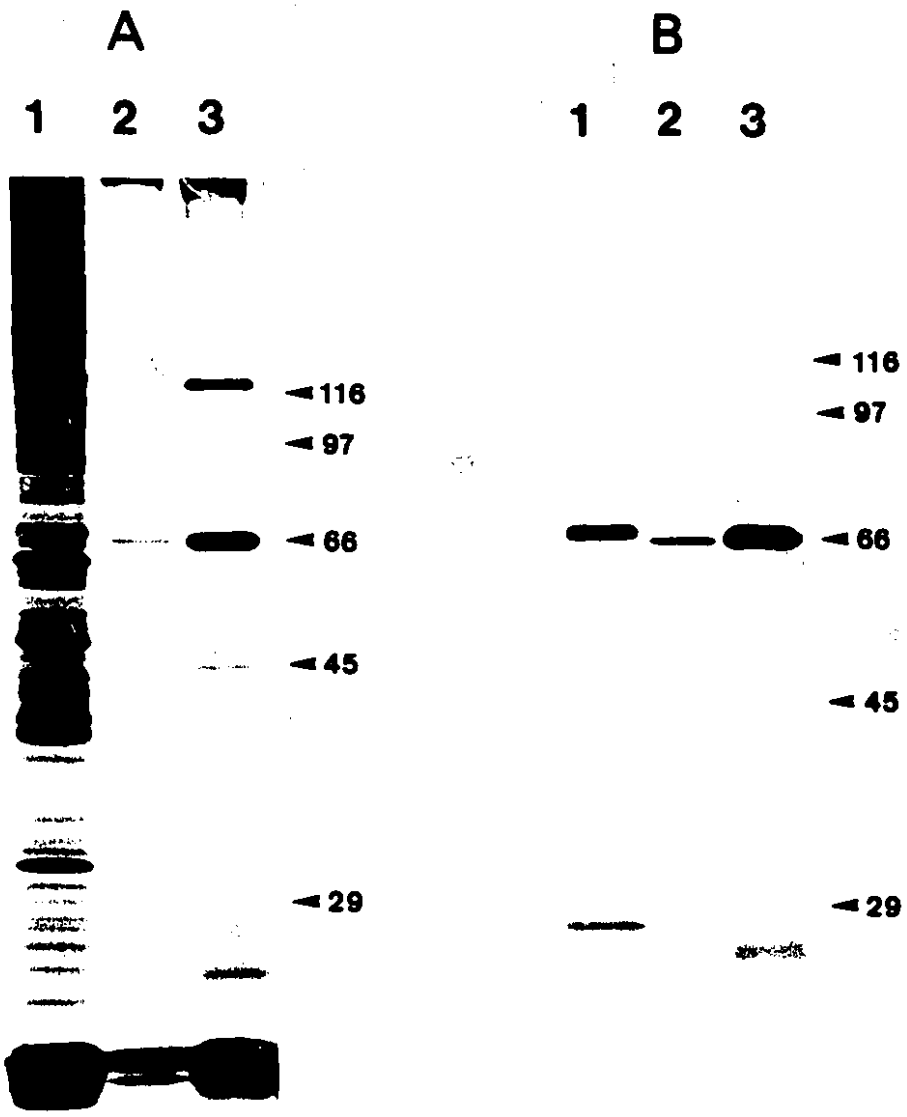
The component polypeptides of acetone:ethanol-washed membrane preparations were electrotransferred from 7-14% concave exponential gradient gels to 0.45 μm nitrocellulose filters in transfer buffer. After reversible staining with Ponceau-S, the blots were processed as described above for the affinity-purification of antibody.

4.4 RESULTS

4.4.1. Specificity of Antibody

Immunoblots were performed to test the validity of screening an expression library from Arabidopsis with antibodies raised against the tonoplast H^+-PPase from Vigna. Tonoplast vesicles were isolated from etiolated hypocotyls of Vigna and storage root of Beta, and microsomes were prepared from seedlings of Arabidopsis. After SDS/PAGE and electrotransfer to nitrocellulose, the membrane preparations were probed with antibody affinity-purified against the 66 kDa subunit of the H^+-PPase from Vigna. The results are shown in Figure 4.1. Immunodetection reveals that only one polypeptide from Arabidopsis reacts strongly with antibody and the polypeptide concerned is of similar apparent size (66.8 kDa) to the corresponding 66 kDa and 64.5 kDa subunits of the H^+-PPase from Vigna and Beta, respectively. Since the antibody employed was affinity-purified against the 66 kDa subunit of Vigna and the same subunit is known, from purification and affinity-labeling studies, to directly participate in substrate- (MgPPi-) binding in both Vigna and Beta (Britten et al., 1989; Rea et al., 1992a), the immune reactions observed are inferred to be H^+-PPase-specific.

Figure 4.1. **Immunoblot analysis of membranes prepared from Arabidopsis, Beta and Vigna and probed with antibody affinity-purified against the 66 kDa subunit of the tonoplast H^+-PPase from Vigna.** A. SDS-gel stained for protein by method of Dzandu et al (1984). B. Immunoblot after reaction with antibody. Lanes 1, Arabidopsis microsomes (20 μg); lanes 2, Beta tonoplast vesicles (8 μg); lanes 3, Vigna tonoplast vesicles (12 μg). Aliquots of the membrane preparations were subjected to SDS/PAGE and stained or electrotransferred to nitrocellulose and probed with affinity-purified antibody. The molecular weights of the principal immunoreactive bands are 66.8 kDa (Arabidopsis), 64.5 kDa (Beta) and 66 kDa (Vigna), respectively.


A
1
2
3
116
97
66
45
29
B
1
2
3
116
97
66
45
29

4.4.2. Selection of cDNA Clones Encoding H^+-PPase

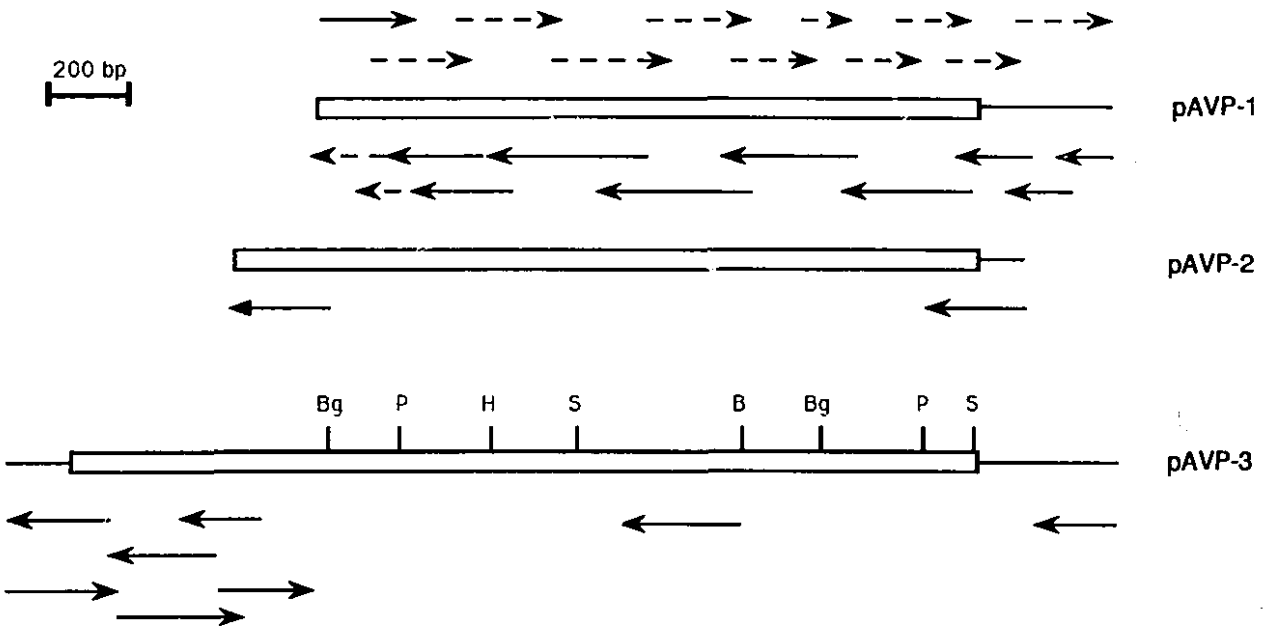
To isolate cDNAs encoding the tonoplast H^+-PPase, the affinity-purified antibody was used to screen a size-fractionated (2-3 kb) Arabidopsis cDNA library constructed in the expression vector λZAP. Approximately 200,000 plaques were screened with antibody and six independent positive clones were obtained. After in vivo excision, the plasmids were found to contain inserts ranging in length from 1.6 to 2.1 kb. Restriction mapping confirmed that all six inserts corresponded to the same gene. Sequence analysis of two of the clones, pAVP-1 and pAVP-2, showed both to contain cDNA inserts of about 2.1 kb with non-overlapping 5′ and 3′ ends (Fig. 4.2).

To obtain longer cDNA inserts encoding the tonoplast H^+-PPase, the cDNA insert of pAVP-1 was used as probe for filter hybridization screens of an Arabidopsis cDNA library constructed in λgt10. From 50,000 plaques, two positive clones containing cDNA inserts of 2.7 and 2.8 kb, respectively, were isolated and subcloned into pBluescriptII. Restriction mapping demonstrated that both clones encode the same gene but contain inserts extending beyond those of pAVP-1 and pAVP-2 in both the 5′ and 3′ directions. The largest λgt10-derived clone, pAVP-3, was sequenced.

4.4.3. Nucleotide Sequence

Both strands of the cDNA insert of pAVP-1 were sequenced completely and additional sequences were obtained from pAVP-2

Figure 4.2. **Sequencing strategy and restriction map of clones pAVP-1, pAVP-2 and pAVP-3.** Dashed arrows indicate inserts sequenced with T3 and T7 primers from nested deletions of pAVP-1. Solid arrows indicate sequences obtained from T3 and T7 primers, or specific, custom-synthesized primers, using complete plasmid clones as templates. The restriction enzymes employed were Bam HI (B), Bgl II (Bg), Hind III (H), Pvu I (PI), Pvu II (PII) and Sca I (S). The insert boundaries demark the Eco RI sites.


200 bp
pAVP-1
pAVP-2
Bg
P
H
S
B
Bg
P
S
pAVP-3

and pAVP-3 (Fig. 4.2) to elucidate the complete nucleotide sequence and predicted amino acid sequence presented in Fig. 4.3A.

The methionine (ATG) codon at position 1 in the nucleotide sequence is identified as the translation initiation site. Only by invoking translation initiation at this point can the direct N-terminal sequence obtained by Maeshima and Yoshida (1989) for the substrate-binding subunit of the H^+-PPase from Vigna (Rea et al., 1992a) be accomodated by the deduced amino acid sequence of the protein encoded by the cDNA. Moreover, only the sequences contiguous with this, the first methionine codon (AAG ATG GT) in the near full-length clone, match the consensus sequence for plant initiator codons (Pu A/C A/C AUG GC) with Pu at -3 and G at +4 being the most critical (Lutcke et al., 1987).

The open reading frame of the cDNA insert of pAVP-3 contains 2310 nucleotides followed by a translation termination codon (TAA) at position 2371 and 348 nucleotides of 3' non-coding sequence (Fig. 4.3A).

4.4.4. mRNA Analysis

The cDNA insert of pAVP-3 is near full-length as indicated by the results of Northern blot analyses (Fig. 4.4). When poly $(A)^+$ RNA is isolated from 7 day-old, dark-grown seedlings of Arabidopsis, size-fractionated and hybridized with random primed AVP-1 cDNA, a single RNA species with an approximate length of 2.8 kb is detected. The lengths of

Figure 4.3. **Nucleotide sequence of Arabidopsis cDNA encoding H^+-PPase and predicted amino acid sequence.** A. Total sequence of cDNA insert of pAVP-3. The underlined amino acids align with the direct sequence data from the MgPPi-binding subunit of the H^+-PPase isolated from Beta and Vigna. B. Alignments of direct sequence data acquired from MgPPi-binding subunit of the tonoplast H^+-PPase isolated from Beta and Vigna with deduced amino acid sequence of Arabidopsis cDNA clone. The sequences of the tryptic fragments derived from the "67 kDa" (Beta-1) and "64.5 kDa" (Beta-2) polypeptides of the H^+-PPase purified from Beta by the methods of Sarafian and Poole (1989) and Britten et al. (1989), respectively, were obtained after SDS/PAGE and electrotransfer to nitrocellulose. In situ tryptic digestion and gas-phase analysis of the fragments after separation by narrow-bore reverse-phase HPLC were performed as described by Aebersold et al (1987). The N-terminal sequence data for the enzyme from Vigna were taken from Maeshima and Yoshida (1989).

A

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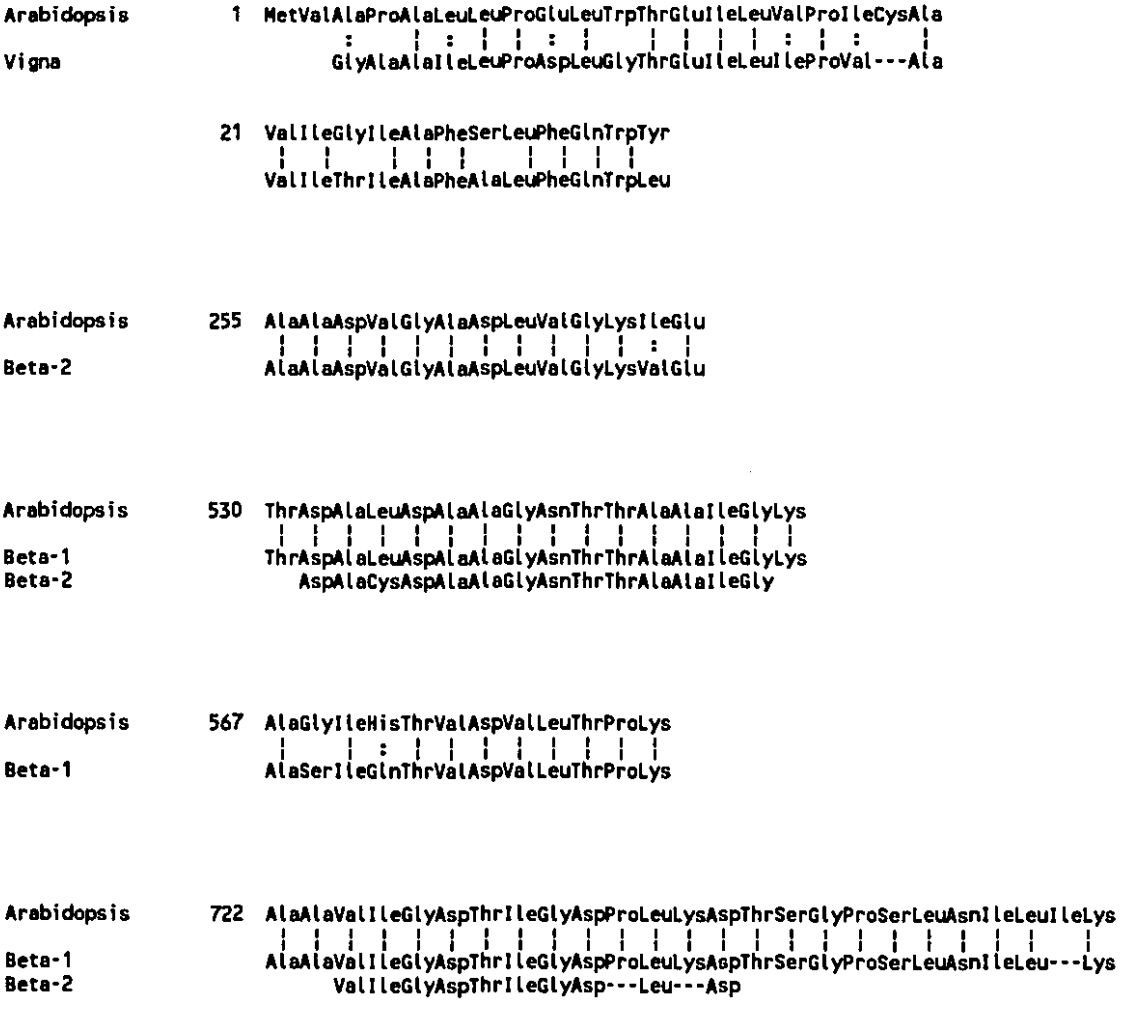
-155      CTTAGATTTATCTTTGAGTCCCGAAACATCGAGGAACGCCTTCGAATCCCTCTCTCTCTGTGTGTGTTCTCTGTGTTCTCTCTCTCGCG   -67
 -66  CGAAGCGGTTCTCTTTCTTTTGTTTATTTGTTTTTATTTGTTTTTCTCTTATACGGAGGAGAGAAGATGGTGGCGCCTGCTTTGTTACCGGAG    27
                                                                    1-MetValAlaProAlaLeuLeuProGlu     9
   28  CTCTGGACGGAGATCCTTGTACCGATTTGTGCGGTGATTGGTATCGCCTTTTCGCTTTTCCAATGGTACGTTGTATCTCGCGTGAAACTCACC   120
   10  LeuTrpThrGluIleLeuValProIleCysAlaValIleGlyIleAlaPheSerLeuPheGlnTrpTyrValValSerArgValLysLeuThr    40
  121  TCTGACCTCGGCGCATCGTCTTCCGGTGGAGCTAACAATGGGAAGAATGGATACGGTGATTATCTAATCGAGGAAGAGGAAGGTGTTAATGAC   213
   41  SerAspLeuGlyAlaSerSerSerGlyGlyAlaAsnAsnGlyLysAsnGlyTyrGlyAspTyrLeuIleGluGluGluGluGlyValAsnAsp    71
  213  CAGAGTGTTGTCGCTAAGTGCGCTGAGATTCAGACTGCTATTTCCGAAGGTGCAACTTCATTCCTATTCACGGAGTACAAATATGTTGGTGTC   306
   72  GlnSerValValAlaLysCysAlaGluIleGlnThrAlaIleSerGluGlyAlaThrSerPheLeuPheThrGluTyrLysTyrValGlyVal   102
  307  TTCATGATTTTCTTTGCTGCTGTTATCTTTGTTTTCCTCGGCTCTGTTGAGGGATTCAGCACTGATAACAAGCCTTGTACTTACGACACCACC   399
  103  PheMetIlePhePheAlaAlaValIlePheValPheLeuGlySerValGluGlyPheSerThrAspAsnLysProCysThrTyrAspThrThr   133
  400  AGAACCTGCAAGCCTGCATTGGCTACTGCAGCTTTCAGTACCATTGCTTTCGTGCTTGGTGCTGTTACCTCTGTTCTATCTGGTTTCCTTGGG   492
  134  ArgThrCysLysProAlaLeuAlaThrAlaAlaPheSerThrIleAlaPheValLeuGlyAlaValThrSerValLeuSerGlyPheLeuGly   164
  493  ATGAAGATTGCTACATACGCTAATGCTAGGACCACTTTGGAGGCGAGGAAAGGTGTTGGAAAGGCGTTCATTGTTGCATTCAGGTCTGGTGCT   585
  165  MetLysIleAlaThrTyrAlaAsnAlaArgThrThrLeuGluAlaArgLysGlyValGlyLysAlaPheIleValAlaPheArgSerGlyAla   195
  586  GTGATGGGTTTCCTTCTTGCAGCGAGTGGTCTATTGGTGCTTTACATTACTATCAATGTGTTCAAGATCTATTACGGAGATGACTGGGAAGGT   678
  196  ValMetGlyPheLeuLeuAlaAlaSerGlyLeuLeuValLeuTyrIleThrIleAsnValPheLysIleTyrTyrGlyAspAspTrpGluGly   226
  679  CTTTTTGAGGCTATTACTGGTTATGGTCTTGGTGGGTCTTCCATGGCTCTCTTTGGCCGTGTTGGTGGTGGGATCTACACTAAGGCTGCTGAT   771
  227  LeuPheGluAlaIleThrGlyTyrGlyLeuGlyGlySerSerMetAlaLeuPheGlyArgValGlyGlyGlyIleTyrThrLysAlaAlaAsp   257
  772  GTCGGCGCTGACCTTGTCGGTAAAATTGAGAGGAATATTCCAGAGGATGATCCAAGAAACCCAGCTGTCATTGCTGATAATGTCGGTGACAAT   864
  258  ValGlyAlaAspLeuValGlyLysIleGluArgAsnIleProGluAspAspProArgAsnProAlaValIleAlaAspAsnValGlyAspAsn   288
  865  GTTGGTGACATTGCTGGTATGGGATCTGATCTCTTTGGATCATATGCTGAAGCATCATGCGCTGCTCTTGTTGTTGCCTCGATCTCATCTTTC   957
  289  ValGlyAspIleAlaGlyMetGlySerAspLeuPheGlySerTyrAlaGluAlaSerCysAlaAlaLeuValValAlaSerIleSerSerPhe   319
  958  GGAATCAACCACGACTTCACTGCCATGTGCTACCCATTGCTCATCAGTTCAATGGGAATCTTGGTTTGTTTGATCACAACTCTCTTTGCCACT  1050
  320  GlyIleAsnHisAspPheThrAlaMetCysTyrProLeuLeuIleSerSerMetGlyIleLeuValCysLeuIleThrThrLeuPheAlaThr   350
 1051  GACTTCTTTGAGATTAAGCTTGTCAAGGAGATTGAACCAGCATTGAAGAACCAGCTCATTATCTCAACTGTTATTATGACTGTTGGTATTGCT  1143
  351  AspPhePheGluIleLysLeuValLysGluIleGluProAlaLeuLysAsnGlnLeuIleIleSerThrValIleMetThrValGlyIleAla   381
 1144  ATTGTGTCATGGGTTGGCTTACCGACCTCCTTTACCATCTTCAACTTTGGAACACAAAAAGTTGTCAAGAACTGGCAGCTATTCCTTTGTGTT  1236
  382  IleValSerTrpValGlyLeuProThrSerPheThrIlePheAsnPheGlyThrGlnLysValValLysAsnTrpGlnLeuPheLeuCysVal   412
 1237  TGTGTTGGTCTTTGGGCTGGACTCATTATTGGTTTCGTCACTGAGTACTACACTAGTAACGCCTACAGCCCTGTGCAAGATGTTGCAGATTCA  1329
  413  CysValGlyLeuTrpAlaGlyLeuIleIleGlyPheValThrGluTyrTyrThrSerAsnAlaTyrSerProValGlnAspValAlaAspSer   443
 1330  TGCAGAACTGGTGCAGCTACCAATGTTATCTTCGGCCTTGCTCTTGGTTACAAATCCGTCATTATTCCAATCTTTGCTATTGCTATCAGTATA  1422
  444  CysArgThrGlyAlaAlaThrAsnValIlePheGlyLeuAlaLeuGlyTyrLysSerValIleIleProIlePheAlaIleAlaIleSerIle   474
 1423  TTCGTTAGCTTCAGCTTTGCTGCTATGTATGGTGTTGCTGTTGCTGCTCTTGGTATGCTCAGTACCATTGCCACTGGTTTGGCAATTGATGCT  1515
  475  PheValSerPheSerPheAlaAlaMetTyrGlyValAlaValAlaAlaLeuGlyMetLeuSerThrIleAlaThrGlyLeuAlaIleAspAla   505
 1516  TATGGTCCCATCAGTGACAATGCTGGTGGTATTGCTGAAATGGCTGGAATGAGCCACCGCATCCGTGAAAGAACTGATGCTCTTGATGCCGCT  1608
  506  TyrGlyProIleSerAspAsnAlaGlyGlyIleAlaGluMetAlaGlyMetSerHisArgIleArgGluArgThrAspAlaLeuAspAlaAla   536
 1609  GGAAACACCACTGCTGCTATTGGAAAGGGATTTGCCATTGGCTCTGCTGCCCTAGTCTCCTTGGCTCTCTTTGGTGCCTTTGTGAGCCGTGCA  1701
  537  GlyAsnThrThrAlaAlaIleGlyLysGlyPheAlaIleGlySerAlaAlaLeuValSerLeuAlaLeuPheGlyAlaPheValSerArgAla   567
 1702  GGGATCCACACCGTAGATGTTTTGACCCCTAAAGTTATCATTGGGCTCCTTGTTGGTGCCATGCTTCCTTACTGGTTCTCTGCCATGACAATG  1794
  568  GlyIleHisThrValAspValLeuThrProLysValIleIleGlyLeuLeuValGlyAlaMetLeuProTyrTrpPheSerAlaMetThrMet   598
 1795  AAGAGTGTGGGAAGTGCAGCTCTTAAGATGGTTGAAGAAGTTCGCAGGCAGTTCAACACCATCCCTGGACTTATGGAAGGAACCGCAAAACCA  1887
  599  LysSerValGlySerAlaAlaLeuLysMetValGluGluValArgArgGlnPheAsnThrIleProGlyLeuMetGluGlyThrAlaLysPro   629
 1888  GACTACGCCACATGTGTCAAGATCTCCACCGATGCTTCCATCAAGGAAATGATACCTCCTGGTTGCCTTGTCATGCTCACACCTCTCATTGTT  1980
  630  AspTyrAlaThrCysValLysIleSerThrAspAlaSerIleLysGluMetIleProProGlyCysLeuValMetLeuThrProLeuIleVal   660
 1981  GGTTTCTTCTTTGGAGTTGAGACCCTCTCTGGTGTCCTCGCCGGATCTCTTGTATCCGGTGTTCAGATCGCCATATCAGCATCTAACACTGGT  2073
  661  GlyPhePhePheGlyValGluThrLeuSerGlyValLeuAlaGlySerLeuValSerGlyValGlnIleAlaIleSerAlaSerAsnThrGly   691
 2074  GGTGCCTGGGACAACGCCAAGAAATACATCGAGGCTGGTGTATCAGAGCACGCAAAGAGCCTTGGACCAAAGGGTTCAGAGCCACACAAGGCA  2166
  692  GlyAlaTrpAspAsnAlaLysLysTyrIleGluAlaGlyValSerGluHisAlaLysSerLeuGlyProLysGlySerGluProHisLysAla   722
 2167  GCTGTGATTGGAGACACAATTGGAGACCCATTGAAGGATACTTCAGGACCTTCATTGAACATCCTCATCAAGCTCATGGCTGTTGAGTCTCTT  2259
  723  AlaValIleGlyAspThrIleGlyAspProLeuLysAspThrSerGlyProSerLeuAsnIleLeuIleLysLeuMetAlaValGluSerLeu   753
 2260  GTCTTTGCTCCCTTCTTCGCCACTCACGGTGGTATCCTTTTCAAGTACTTCTAAACTCAATCCGAGGGAAGAAGATGACGATGATGAAGAAGA  2352
  754  ValPheAlaProPhePheAlaThrHisGlyGlyIleLeuPheLysTyrPhe-770
 2353  AGAAGATGATGATGGCGATCGATTCTAAACTTTCTTTTTTACCATTCTTATTTTCGTTTACCGTAGGTGGTTAAAAAACCTTTTTGTTGATGA  2445
 2446  GGCTCATTTAAAGAACCAACCAAATGATGTTTCTTTCTCTCACTCTCTGTCTTTCTGTTTTCTTTTTGTTCTGTTTAGAATTTAGAAATCCAC  2538
 2539  CAAGTATTCGGTCGAGACTTGTTTTAGCCGTTACTTTCTGCTGCTTATATTTCCTAAATTGGTTGTCTTCTTCGAAACATAATTGGAATTTAT  2631
 2632  TGTTACTGTTAGTCTAAAAAAAAAAAA  2658
```

B


Arabidopsis 1 MetValAlaProAlaLeuLeuProGluLeuTrpThrGluIleLeuValProIleCysAla
Vigna GlyAlaAlaIleLeuProAspLeuGlyThrGluIleLeuIleProVal---Ala
21 ValIleGlyIleAlaPheSerLeuPheGlnTrpTyr
ValIleThrIleAlaPheAlaLeuPheGlnTrpLeu
Arabidopsis 255 AlaAlaAspValGlyAlaAspLeuValGlyLysIleGlu
Beta-2 AlaAlaAspValGlyAlaAspLeuValGlyLysValGlu
Arabidopsis 530 ThrAspAlaLeuAspAlaAlaGlyAsnThrThrAlaAlaIleGlyLys
Beta-1 ThrAspAlaLeuAspAlaAlaGlyAsnThrThrAlaAlaIleGlyLys
Beta-2 AspAlaCysAspAlaAlaGlyAsnThrThrAlaAlaIleGly
Arabidopsis 567 AlaGlyIleHisThrValAspValLeuThrProLys
Beta-1 AlaSerIleGlnThrValAspValLeuThrProLys
Arabidopsis 722 AlaAlaValIleGlyAspThrIleGlyAspProLeuLysAspThrSerGlyProSerLeuAsnIleLeuIleLys
Beta-1 AlaAlaValIleGlyAspThrIleGlyAspProLeuLysAspThrSerGlyProSerLeuAsnIleLeu---Lys
Beta-2 ValIleGlyAspThrIleGlyAsp---Leu---Asp

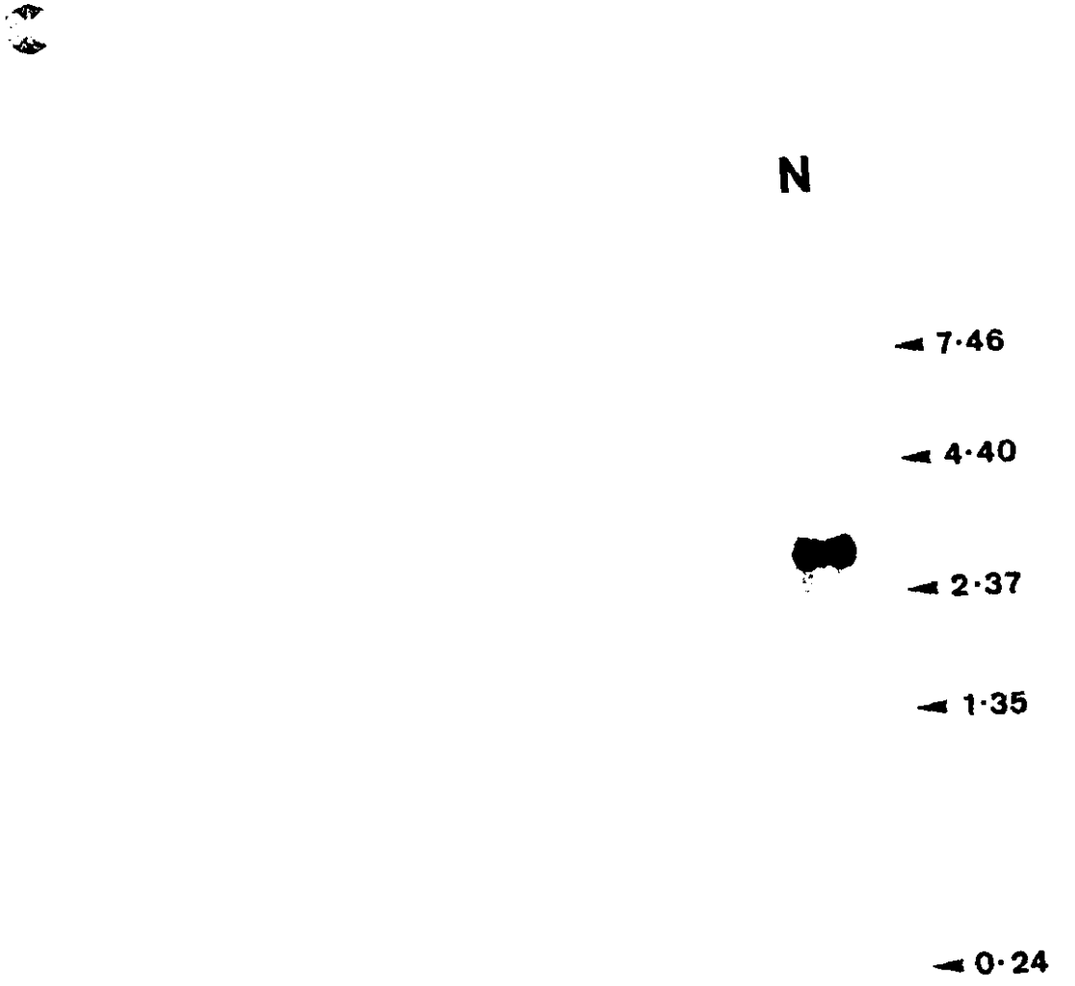
AVP-3 and mature Arabidopsis transcript therefore coincide.

4.4.5. Identity of cDNA Clones

The identity of the putative H^+-PPase cDNA clones from Arabidopsis is verified by the precise alignment of the deduced amino acid sequence of the protein encoded by AVP-3 with the direct internal sequence data acquired from the 64.5-67 kDa, MgPPi-binding polypeptide of Beta (Sarafian & Poole, 1989; Britten et al., 1989) and the N-terminal sequence data obtained from the corresponding polypeptide of Vigna (Maeshima & Yoshida, 1989).

In situ tryptic digestion (Aebersold et al., 1987) of the "67 kDa" (Beta-1) and "64.5 kDa" (Beta-2) substrate-binding subunits of the H^+-PPase purified by the methods of Sarafian and Poole (1989) and Britten et al., (1989), respectively, generates peptide fragments with identical sequences (Fig. 4.3B). Of a total of 6 tryptic fragments subjected to gas-phase sequence analysis, three from Beta-1 and three from Beta-2, two fragments from each preparation overlap exactly with two fragments from the other preparation (Fig. 4.3B). Moreover, all six stretches of sequence, including those unique to the two Beta H^+-PPase preparations, are present in the open reading frame of clone pAVP-3 at positions 255, 530, 567 and 722, respectively, in the deduced amino acid sequence. The direct sequence from the 64.5-67 kDa subunit of Beta and the amino acid sequence deduced from the nucleotide sequence of pAVP-3 show complete identity over a total span

Figure 4.4. **Northern blot analysis (N) of Arabidopsis tonoplast H^+-PPase.** One μg of poly $(A)^+$ RNA isolated from 7 day-old, dark-grown Arabidopsis seedlings was electrophoresed, blotted and probed with radiolabeled cDNA insert AVP-1. The positions of the RNA molecular size markers (kb) are indicated.

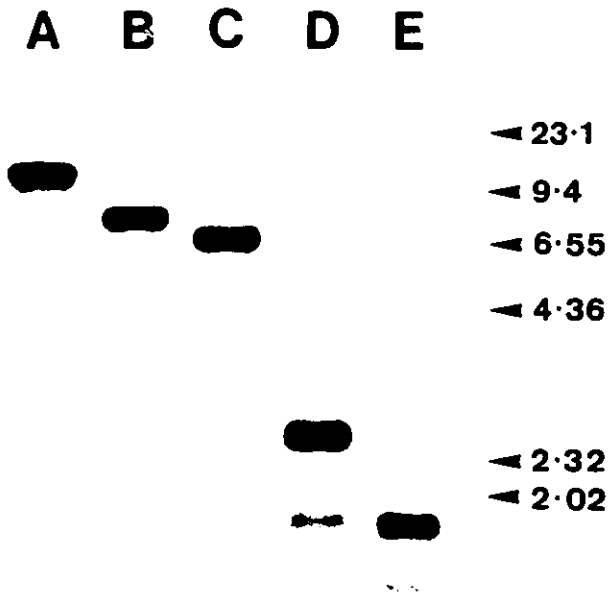

N
7·46
4·40
2·37
1·35
0·24

of 66 amino acid residues except for two conservative (Val -> Ile, Gln -> His) substitutions at positions 266 and 570 and one non-conservative (Ser -> Gly) substitution at 568. Comparison of the deduced N-terminal sequence of the open reading frame of the cDNA insert of pAVP-3 and the N-terminus of the substrate-binding subunit of the H^+-PPase from Vigna, on the other hand, reveals 19 identities and 5 conservative substitutions within a span of 30 amino acid residues starting at position 3 (Fig. 4.3B). These findings not only demonstrate that the open reading frame of the cDNA insert of pAVP-3 (and pAVP-1 and pAVP-2) encodes the substrate-binding subunit of the tonoplast H^+-PPase but also show, in agreement with the immunological data (Fig. 4.1), that this subunit exhibits a high degree of sequence conservation between Arabidopsis, Beta and Vigna.

4.4.6. Number of Tonoplast H^+-PPase Genes in Arabidopsis

A Southern blot analysis of Arabidopsis genomic DNA, using the 2.1 kb cDNA insert of pAVP-1 as radioactive probe was performed to enumerate the genes which encoded the substrate-binding subunit of the H^+-PPase in this organism (Fig. 4.5). Of the five enzymes chosen for the genomic digests, two (Hind III and Bgl II) each have one restriction site within the 2.1 kb fragment while the other three (Xba I, Eco RV and Eco RI) do not (Fig. 4.2). Thus, providing that the region, or regions, of the genome corresponding to the 2.1 kb fragment are devoid of introns containing the same

Figure 4.5. **Genomic Southern analysis of tonoplast H^+-PPase.** Arabidopsis genomic DNA digested with Xba I (lane A), Eco RV (lane B), Eco RI (lane C), Hind III (lane D) or Bgl II (lane E) was electrophoresed, blotted and hybridized with ^{32}P-labeled AVP-1. The positions of the DNA molecular weight markers (kb) are indicated.


A
B
C
D
E
23·1
9·4
6·55
4·36
2·32
2·02

restriction sites, a single band from the Xba I, Eco RV and Eco RI digests and two bands from each of the Hind III and Bgl II digests would be expected if the H^+-PPase is coded by a single gene. This is exactly what is found. Genomic DNA digested with Xba I, Eco RV and Eco RI, respectively, and probed with the 2.1 kb fragment yields intensely hybridizing bands at 7, 8 and 12 kb, respectively. The Hind III and Bgl II digests, on the other hand, each contain two hybridizing bands at 2.6 and 1.83 kb and 1.78 and 1.4 kb, respectively. If there are multiple genes for the H^+-PPase they are sufficiently divergent for their corresponding nucleotide sequences not to cross-hybridize under the conditions employed.

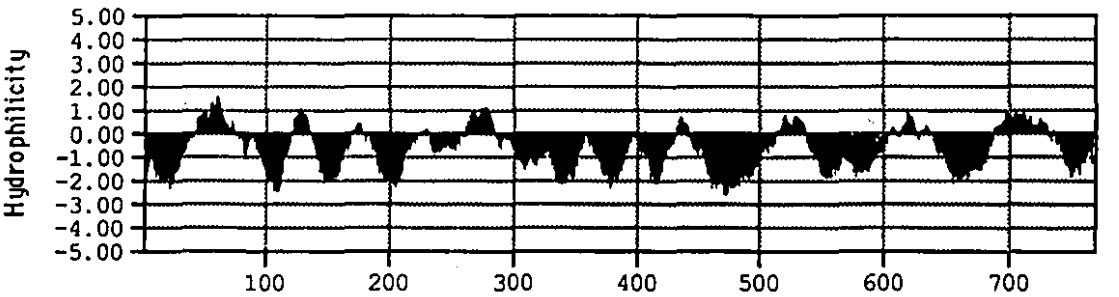
4.4.7. Deduced Amino Acid Sequence

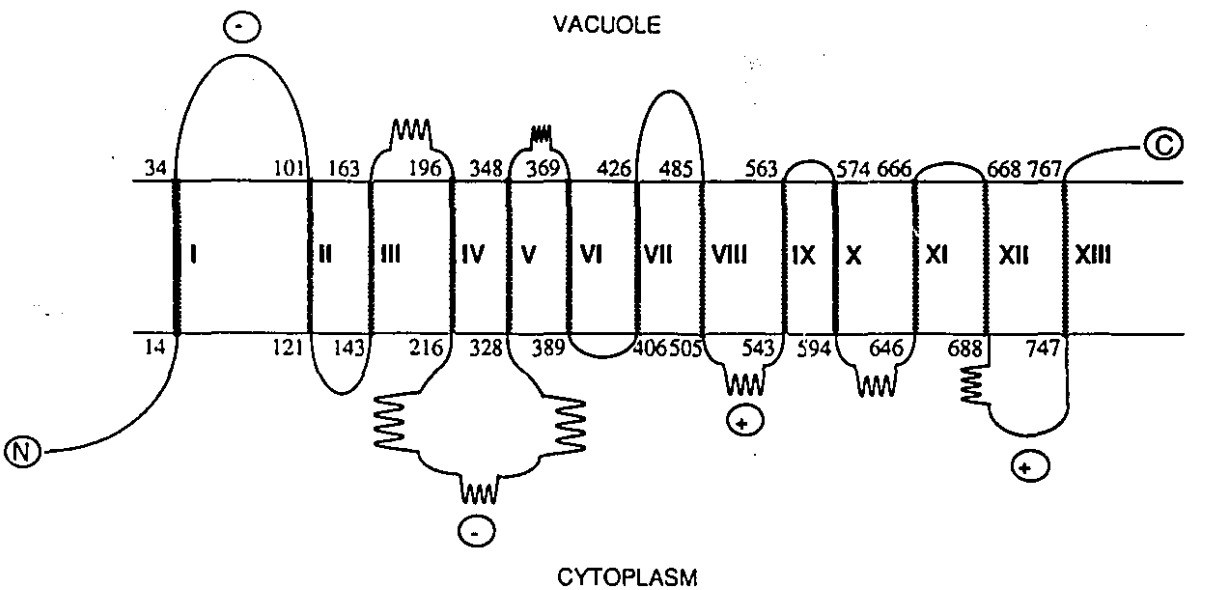
The open reading frame of AVP-3 encodes a 770 amino acid polypeptide with a pI of approximately 4.95 and a predicted molecular weight of 80,800 daltons ("81 kDa"). While the estimated molecular weight of the protein encoded by the insert is approximately 8 kDa larger than the highest apparent (73 kDa) determined by SDS/PAGE of the enzyme from *Vigna* (Maeshima & Yoshida, 1989), 13.8-16.3 kDa greater that the estimated size (64.5-67 kDa) of the corresponding subunit from *Beta* (Britten *et al.*, 1989; Sarafian & Poole, 1989) and about 14 kDa greater than the 66.8 kDa immunoreactive polypeptide reported here for *Arabidopsis* (Fig. 4.1), the anomalous migration of hydrophobic membrane proteins on SDS

gels is not unusual. The binding of non-saturating amounts of SDS by membrane proteins is common and frequently accompanied by large shifts in apparent molecular weight as a result of the exposure of charged amino acid residues and/or irregularities in the shape of the SDS-protein complex (Maddy, 1976).

Computer-assisted hydropathy plots of the Arabidopsis H^+-PPase amino acid sequence establish that the cDNA insert of pAVP-3 encodes an extremely hydrophobic, integral membrane protein (Fig. 4.6). Membrane-associated α-helices were determined by hydrophobic moment analysis (Eisenberg et al., 1984) and some of the secondary structural characteristics of the hydrophilic sequences was assigned by the Garnier method (Garnier et al., 1978). The model contains 13 transmembrane spans, all of which are amphipathic ("multimeric") and may interact to stabilize the structure through the formation of interhelical H-bonds and salt-bridges. In addition, several of the hydrophilic domains are characterized by the presence of clusters of charged residues. Thus, the hydrophilic segment linking membrane spans I and II contains 4 contiguous Glu residues (positions 64-67), the segment linking spans IV and V contains 4 acidic residues in a stretch of eight amino acids (positions 222-229), the segment between spans VIII and IX contains the sequence ArgXArgXArg (positions 525-529) and the penultimate hydrophilic domain, linking spans XII and XIII, contains a preponderance of Lys residues. The overall orientation of the 81 kDa polypeptide is depicted as shown in Figure 4.6 in accord with the "positive-inside rule" (von

Figure 4.6. Computer-assisted hydrophilicity plot and topographic model of *Arabidopsis* tonoplast H^+-PPase amino acid sequence. The plots were calculated according to Kyte and Doolittle (1982) with the MacVector program over a running window of 20 amino acid residues. Values above the median represent hydrophilic segments; values below the median represent hydrophobic segments. The numbers I through XIII represent the putative membrane-spanning segments depicted in the tentative topographic model of the tonoplast H^+-PPase. Transmembrane segments, all of which appear to be multimeric on the basis of their hydrophobic moment, were predicted by the HELIXMEM program of PC/GENE. The structure of the non-transmembrane regions was examined according to the secondary structure predictions of Garnier *et al*. (1978) using both the GARNIER and GGBSM programs of PC/GENE. The structures indicated are: /\/\/\ , α-helix; ------ , random coil; - , clusters of negative charge; + , clusters of positive charge; N, amino-terminus; C, carboxy-terminus.


Hydrophilicity
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
100
200
300
400
500
600
700


VACUOLE
34
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348
369
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485
563
574
666
668
767
I
II
III
IV
V
VI
VII
VIII
IX
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XI
XII
XIII
14
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143
216
328
389
406
505
543
594
646
688
747
N
C
CYTOPLASM

Heine & Gavel, 1988) wherein, for the majority of multiply spanning membrane proteins, most of the positively charged amino acids are disposed towards the cytoplasmic face of the membrane.

Database searches of the H^+-PPase sequence confirm the novelty of this pump. Computer searches of both the nucleotide sequence of the cDNA insert of pAVP-3 and the deduced amino acid sequence of the polypeptide encoded by the clone against the GENBANK, GENPEPT and Swiss Protein databases yielded no detectable homology between this pump and any other sequenced ion translocase or PPi-dependent enzyme.

4.5. CONCLUSIONS

The MgPPi-binding subunit of the tonoplast H^+-PPase appears to be the sole polypeptide constituting the functional enzyme complex. The component, alone, copurifies with PPase activity during detergent-solubilization and chromatography (Maeshima & Yoshida, 1989; Britten et al., 1989; Sarafian & Poole, 1989) and is the only polypeptide of tonoplast vesicles susceptible to MgPPi-protectable, free PPi-potentiated labeling by [^{14}C]-N-ethylmaleimide (Britten et al., 1989). Accordingly, selective purification of the MgPPi-binding subunit of the tonoplast H^+-PPase from Vigna and its incorporation into artificial liposomes results in the reconstitution of both MgPPi hydrolysis and MgPPi-dependent H^+-translocation (Britten et al., 1992).

Together with the amino acid sequence data derived from pAVP-3, which show that the MgPPi-binding subunit of the tonoplast H^+-PPase is highly hydrophobic and possesses multiple transmembrane spans, these findings strongly suggest that subunits in addition to the 81 kDa polypeptide encoded by AVP-3 need not be implicated to account for the capacity of the tonoplast H^+-PPase for PPi-energized transtonoplast H^+-translocation. This conclusion is corroborated by a recent estimate of the functional mass of the tonoplast H^+-PPase during substrate hydrolysis which yielded a value of 91 kDa (Sarafian et al., 1992b) in agreement with our deduced subunit mass of 81 kDa for the protein encoded by AVP-3. Though the H^+-PPase may function as an oligomer during proton translocation (Sarafian et al., 1992b), the data available do not contradict the possibility that we have cloned the H^+-PPase in its entirety.

Subcellular fractionation of plant cells suggests that the tonoplast H^+-PPase, like the V-type, tonoplast H^+-ATPase, coresident on the same membrane (Rea & Sanders, 1987), is present in at least two organellar membrane systems: those bounding the vacuole (Rea & Poole, 1985; Wang et al., 1986) and those constituting the Golgi complex (Chanson et al., 1985). The results from our molecular investigations complement and extend the results deriving from these studies to indicate that the same polypeptide mediates PPi-dependent H^+-translocation in both systems. Since the gene encoding the tonoplast H^+-PPase is present in a single copy in the genome

of Arabidopsis and it is probable that the labeled probe used for the genomic hybridizations would detect a second highly conserved H^+-PPase gene in the same organism, it is likely that the gene detected encodes the H^+-PPase of both vacuolar and Golgi membranes.

On the basis of the results reported here and what is known of its subunit composition, substrate-specificity and inhibitor-sensitivity, the tonoplast H^+-PPase must be ascribed to a completely new category of H^+-translocase. Sequence identities with any characterized H^+-ATPase are lacking, the type-specific inhibitors azide, orthovanadate and bafilomycin, which selectively and strongly inhibit F-, P- and V-type H^+-ATPases, respectively, are without effect on the tonoplast H^+-PPase (Rea & Sanders, 1987) and none of the subunits of F-, P- and V-type ATPases are immunologically cross-reactive with the MgPPi-binding subunit of the enzyme (Rea, P.A., unpublished; Sarafian, V. and Poole, R.J., unpublished).

Close phylogenic links between the tonoplast H^+-PPase and soluble PPases are also unlikely. All known soluble PPases have subunit sizes different from the tonoplast H^+-PPase - 20 kDa for the enzymes from prokaryotes (Lahti et al., 1988) and 32-42 kDa for the enzymes from eukaryotes (e.g. Cohen et al., 1978; Krishnan & Gnanam, 1988) - and none of the known sequences for soluble PPases (Arabidopsis, Kieber & Signer, 1991; E. coli, Lahti et al., 1988; Kluyveromyces lactis, Stark & Milner, 1989; Saccharomyces cerevisiae,

Kulakowski et al., 1988) align with the deduced sequence of the tonoplast H^+-PPase. Similarly, the recently cloned catalytic 28-30 kDa subunit of the membrane-bound mitochondrial H^+-PPase (Lundin et al., 1991) may be eliminated as a homolog: the subunit from Saccharomyces is 49% identical to the soluble PPase from the same source (Lundin et al., 1991), shows no sequence identities with the tonoplast H^+-PPase from Arabidopsis, and the corresponding enzyme from rat liver mitochondria does not cross-react with antibody raised against the tonoplast H^+- PPase from Vigna (Maeshima, 1991).

A cautionary note is, however, warranted. Direct sequence comparisons between the tonoplast H^+-PPase and other known PPases are complicated by the fact that clones of the soluble PPases from a range of organisms including Arabidopsis, E. coli, Kluyveromyces, Saccharomyces and Schizosaccharomyces (Lahti et al., 1990) demonstrate only modest overall sequence identities of 20-27% (Lahti et al., 1990), but functionally important residues are nonetheless conserved. Crystallographic studies of the soluble PPase from Saccharomyces have identified 17 residues thought to be involved in Mg^{2+} and PPi binding and 11-16 of these residues (depending on alignment procedure) are conserved between the enzymes from Arabidopsis, E. coli, Kluyveromyces, Saccharomyces and Schizosaccharomyces (Lahti et al., 1990). Eight of the active site residues of soluble PPases fall into two configurations - EXXXXXXXKXE and DEXEXDXKXXXXD - at positions 48 and 146, respectively, in the sequence of the enzyme from Saccharomy-

ces (Lahti et al., 1990). Significant therefore is the presence of variants of these two motifs - DXXXXXXXKXE and DXXXXDXKXXXXD, respectively - at positions 257 and 119, respectively, in the deduced amino acid sequence of the Arabidopsis tonoplast H^+-PPase (Fig. 4.3A). If the gross orientation of the tonoplast H^+-PPase is as depicted in Figure 4.6, the motif DXXXXDXKXXXXD lies within the second hydrophilic domain connecting transmembrane spans II and III whereas the motif DXXXXXXXKXE lies within the fourth extramembranous loop connecting spans IV and V. Consequently, both motifs would be expected to be located on the cytoplasmic face of the membrane as are the substrate and divalent cation binding sites of the tonoplast H+-PPase. Moreover, because the putative MgPPi-binding motif at position 119 contains one Cys residue and is immediately flanked by another, it is tempting to speculate that the pronounced sensitivity of the tonoplast H^+-PPase to MgPPi-protectable, free PPi-potentiated inhibition and covalent modification by the sulfhydryl reagent N-ethylmaleimide (Britten et al., 1989) is attributable to alkylation of one or both of these Cys residues. Thus, while the toncplast H^+-PPase and soluble PPases appear to be remote evolutionarily, they may share convergent sequence motifs deriving from the fact that both classes of enzyme must interact with the same substrates and cofactors.

Evaluation of a potentially more promising evolutionary relationship between the tonoplast H^+-PPase and the revers-

ible H^+-translocating PPase (H^+-PPi synthase) of phototrophic bacteria will be contingent on the acquisition of sequence data from the latter category of enzyme. The existence of a H^+-PPi synthase on the energy-coupling membranes of phototrophic bacteria, notably Rhodosprillum rubrum, has been known for some time (Baltscheffsky & Nyren, 1987), but only recently has it been shown that this translocase is an integral membrane protein with an apparent molecular size of 56 kDa (Nyrén et al., 1991). Two features of the 56 kDa polypeptide are significant: it is immunologically cross-reactive with the MgPPi-binding subunit of the tonoplast H^+-PPase (Kim, E.J., Kim, Y. and Rea, P.A., unpublished) and, unlike the 28-30 kDa peripheral catalytic subunit of the mitochondrial H^+-PPase (Lundin et al., 1991), the 56 kDa subunit, alone, is capable of mediating both MgPPi hydrolysis and H^+-translocation (Nyrén et al., 1991). Homology between the H^+-PPases from Rhodospirillum and plant vacuoles is consequently a strong possibility in which case this category of H^+-translocase may be as ancient as the V- and F-type H^+-ATPases (Nelson & Taiz, 1989). Genomic screens of Rhodospirillum using the cDNA insert of pAVP-1, and derivatives thereof, as probes are currently in progress to examine the validity of this prediction.

To our knowledge, the findings reported here represent the first isolation and sequence analysis of any vacuolar H^+-PPase clone. As such, they make this unique H^+ pump amenable to detailed genetic analyses for the first time. The

full physiological significance of the tonoplast H^+-PPase *in vivo* might therefore be addressed by gene transformation techniques. Two overriding questions are the involvement of the tonoplast H^+-PPase in cellular PPi regulation and vacuolar K^+ transport. A remarkable characteristic of cellular PPi levels in plants is their invariancy. PPi levels do not change during light-dark transitions (Weiner *et al.*, 1987), during rapid changes in respiration rate or when tissues are subjected to anoxia or respiratory poisons (Dancer & ap Rees, 1989). The operation of a highly effective PPi-stat is therefore implicated. What are the roles of the tonoplast H^+-PPase and the recently cloned soluble PPase of *Arabidopsis* (Kieber & Signer, 1991) in cellular PPi-stasis and how do the two enzymes interact? At the level of plant mineral nutrition, recent electrophysiological studies on intact, isolated vacuoles strongly suggest that rather than merely acting as a supplementary H^+ pump, the H^+-PPase may also catalyze K^+-H^+ symport into the vacuole (Davies *et al.*, 1991). Might the tonoplast H^+-PPase therefore be instrumental for cell turgor regulation, in general, and stomatal guard cell inflation, in particular? The availability of cDNAs encoding the tonoplast H^+-PPase should now enable the construction of transgenic plants that either express antisense transcripts (van der Krol *et al.*, 1988) of the H^+-PPase gene or ectopically overexpress the gene (Lagrimini *et al.*, 1990) for investigations of the phenotypic consequences of perturbed H^+-PPase gene expression on cellular PPi and K^+ homeostasis.

CHAPTER 5

GENERAL DISCUSSION AND PHYLOGENETIC HYPOTHESIS

5.1. GENERAL DISCUSSION

The work described in this thesis pertains to the molecular characterization of the inorganic pyrophosphate-energized H^+ pump located in the vacuolar membrane of higher plants.

CHAPTER 2 describes the purification procedure which resulted in the identification of the hydrolytic subunit of the H^+-PPase as a 67 kDa polypeptide. The enzyme was detergent-solubilized from the storage root of red beet (Beta vulgaris L) tonoplast vesicles and purified after succesive passages through anion-exchange and size-exclusion chromatography columns. SDS/PAGE revealed a strong correlation between the densities of the 67 kDa polypepetide in consecutive fractions and the activity of the enzyme in the same fractions. Although the purification was not carried to homogeneity, the presence of contaminating proteins was made more visible by the deliberate overload of the SDS gel, which was necessary in order to facilitate the densitometric analysis. Other studies subsequently confirmed that a single polypeptide species of similar molecular weight was sufficient for PPi hydrolysis. A 64 kDa polypeptide was identified as the hydrolytic subunit in Beta by Britten et al., (1989) and the equivalent one from Vigna had a molecular weight of 73 kDa (Maeshima & Yoshida, 1989). A preliminary estimate of the size of the holoenzyme, obtained from size exclusion chromatography, gave an approximate value of 250 kDa.

In CHAPTER 3 estimates of the enzyme's functional sizes, determined by radiation inactivation, are presented. The results indicated that a single subunit is sufficient for substrate hydrolysis, whereas H^+ translocation requires a complex of 300 to 350 kDa. Parallel tests were performed on the vacuolar H^+-ATPase, using the same assay methods, to verify the functional sizes required for substrate hydrolysis and H^+ pumping. The values obtained from both assays ranged from 400 to 500 kDa, discounting a methodological artifact as the reason for the differences in the H^+-PPase sizes.

As a final molecular characterization, the cDNA encoding the H^+-PPase in Arabidopsis thaliana was isolated, cloned and sequenced (CHAPTER 4). Peptide sequences obtained from tryptic fragments of the purified 67 kDa polypeptide from Beta confirmed that the isolated clone coded for the H^+-PPase gene. In addition, the N-terminal sequence of the Vigna H^+-PPase (Maeshima & Yoshida, 1989) matched the putative amino terminus of the Arabidopsis clone. Analysis of the amino acid sequence revealed a highly hydrophobic protein with a molecular weight of 81 kDa. The lack of sequence homologies with any other known polypeptide implies that the H^+-PPase belongs to a new class of proton pumps.

The absence of significant homologies with other ion pumps or with PPi-utilizing enzymes has interesting phylogenetic implications. It is, of course, possible that this enzyme is widely distributed throughout living organisms and is yet to be recognized. It is also possible that it is

restricted to plants, performing a function uniquely adapted to their physiology. However, the presence of a very similar enzyme in Rhodospirillum rubrum, which cross-reacts with antibodies raised against the plant H^+-PPase, implies some evolutionary links which are not immediately apparent since plants and photosynthetic bacteria differ at the highest taxonomic level.

The following section is an (highly speculative) attempt to explain the evolutionary pathway which could account for the presence of the H^+-PPase in plants.

5.2. HYPOTHESIS TO EXPLAIN THE PHYLOGENETIC ORIGINS OF THE H^+-PPASE

5.2.1. Distributions of the vacuolar H^+-ATPase and H^+-PPase in living organisms

It is now well established that plant vacuoles have two proton pumps, a H^+-ATPase and a H^+-PPase. It has also been demonstrated that the vacuolar-type H^+-ATPase is present in fungal vacuoles (Bowman et al., 1986), bovine clathrin-coated vesicles (Xie & Stone, 1986) and chromaffin granules (Percy et al., 1985) as well as in the plasma-membranes of archaebacteria such as Sulfolobus acidocaldarius (Gogarten et al., 1989) and Methanococcus thermolithothropicus (Bernasconi et al., 1989). These various v-type ATPases show remarkable conservation in their subunit composition and high homologies (>50%) in the primary sequences of their corresponding subunits (Manolson, 1988; Gogarten et al., 1989). The similarities among v-type ATPases prompted their use as evolutionary markers (Gogarten et al., 1989) and has aided the determination of phylogenetic distances among the three main evolutionary branches, namely the archaebacteria, the eubacteria and the eukaryotes, as proposed by Woese (1987) and Lake (1988).

In contrast, the vacuolar H^+-PPase has not been shown to be as widely distributed among genetically distant organisms. This is undoubtedly due to the enzyme's relatively recent

discovery, but there are indications that the distribution of the H^+-PPase differs from that of the H^+-ATPase. At present, the only documented occurrences of the H^+-PPase other than in plant vacuoles are in photosynthetic bacteria such as Rhodospirillum rubrum (whose well characterized enzyme cross-reacts with antibodies raised against the plant H^+-PPase; Nore et al., 1991), Rhodopseudomonas palustris (whose chromatophores contain an uncoupler-stimulated PPase; Schwarm et al., 1986) and Rhodopseudomonas viridis (in which light-induced PPi synthesis has been reported; Nore et al., 1990). It is interesting to note that the H^+-PPase is absent from Rhodobacter capsulatus, which belongs to the same group of photosynthetic bacteria as the ones above (Nore et al., 1990).

PPase activity is absent from bovine chromaffin granules (D. Stone, personal communication) and bovine clathrin coated vesicles (P.A. Rea, personal communication) as well as from vacuoles of the yeast Saccaromyces cerevisiae. The latter was assayed both for PPi-induced proton pumping (M. Manolson, personal communication) and for immunological cross-reactivity with plant anti-H^+-PPase antibodies (E. Kim, V. Sarafian and P.A. Rea, unpublished data). Genomic Southern blots of S. pombe and S. carlsbergensis probed with a cDNA encoding the H^+-PPase from Arabidopsis failed to show cross-hybridization (R. Eisman, E.J. Kim and P.A. Rea, personal communication) suggesting that the H^+-PPase reported by Kulakovskaya et al. (1989) in S. carlsbergensis vacuoles is either unrelated to

the plant H^+-PPase or was due to mitochondrial contamination.

5.2.2. Possible evolutionary origins of the H^+-PPase

The α group of purple non-sulfur photosynthetic bacteria, whose representatives include Rhodospirillum and Rhodopseudomonas, are among the most primitive eubacteria known (Woese, 1987). As the loss of light-harvesting systems would intuitively seem more plausible than their independent evolution in a separate line, it has been suggested that the first eubacteria were photosynthetic and that the non-photosynthetic forms lost the ability to utilize the energy of light (Harold, 1986). The H^+-PPase would therefore be as ancient as the earliest eubacteria. The presence of a membrane-bound PPase in non-photosynthetic eubacteria and archaebacteria is poorly documented even though soluble PPases are common and well studied (Lahti, 1983). The possibility of a membrane-bound PPi synthase coupled to methane formation, in the archaebacterium Methanobacterium thermoautotrophicum has been raised (Keltjens et al., 1988). Methane formation and PPi synthesis occured in parallel and were inhibited by a proton-gradient dissipating compound.

Since the H^+-PPase is found in a eubacterial group (Rhodospirillum) and a eukaryotic group (plants), then the enzyme's origins should predate the separation of the two evolutionary lines. It also follows that the H^+-PPase gene should have been retained by the archaebacteria since Lake (1988) and the vacuolar ATPase phylogeny (Gogarten et al.,

1989) suggest that archaebacteria and eukaryotes are more closely related to each other than to eubacteria. It is unlikely that the common ancestor (the progenote, as defined by Woese, 1987) of the three had a H^+-PPase which was retained by one group of eubacteria (the α purple - but not Rhodobacter) and one group of eukaryotes (plants) but not by fungi (which are more primitive than plants; Woese, 1987) or animals. Another possible route to explain the presence of the H^+-PPase in plants is through a direct transfer from a eubacterial organism.

5.2.3. Eubacterial origin of the plant H^+-PPase

The α subdivision of the purple bacteria includes such groups as the agrobacteria and the rhizobacteria which are known to form intimate associations with plants, including DNA transfer (Woese, 1987). A direct genetic transfer to the host's nuclear DNA is possible and would have occurred at the branching point of plants from other eukaryotes since primitive as well as higher plants have the H^+-PPase. Another mode of entry would be through endosymbiotic relationships such as those which are believed to be the origins of chloroplasts and mitochondria (Margulis, 1981).

5.2.4. Gene transfer via chloroplasts

It is now generally accepted that chloroplasts originated in plants through an invasion by a photosynthetic bacterium which formed an endosymbiotic relationship with its

eukaryotic host (Gray, 1988). The invading organism most likely belonged to one of the earliest photosynthetic eubacterial groups endowed with photosystems I and II, the cyanobacteria, since it is unlikely that plants developed such a complex system de novo (Harold, 1986). The evidence linking the cyanobacteria to the origin of chloroplasts was obtained from sequence comparisons of ferredoxin, c-type cytochromes and 5S ribosomal RNA subunits (Schwartz & Dayhoff, 1978) as well as the small (SSU) and large (LSU) rRNA subunits (Cedergren et al., 1988; Van de Peer et al., 1990).

The question which remains unanswered is whether the cyanobacteria possess a H^+-PPase which after the formation of the endosymbiotic coupling would have been transferred to the nucleus of the host. The only available evidence to date for the presence of a membrane-bound PPase in cyanobacteria comes from a study on Anacystis nidulans particles which were shown to be competent for PPi hydrolysis but lacked energy coupling (Bornefeld, 1981).

5.2.5. Gene transfer via mitochondria

The phylogenetic origins of the mitochondria are, like the chloroplasts, believed to be endosymbiotic (Gray, 1989). There are strong homologies between mitochondrial and eubacterial SSU and LSU rRNAs (Yang et al., 1985; Cedergren et al., 1988). Their protein synthesizing mechanisms are very similar, both being inhibited by chloramphenicol and resistant to cycloheximide (Gray, 1988). Phylogenetic trees based

on rRNA sequences have shown close links between mitochondria, especially from plants, and the α purple bacteria (the group to which Rhodospirillum rubrum belongs). Interestingly, the same phylogenetic trees place the mitochondria of other eukaryotes on different branches, considerably more distant from the eubacterial ancestor than the plant mitochondria. The possibility of a polyphyletic origin of mitochondria has also been raised (Gray et al., 1989; Van de Peer et al., 1990). However, there is a consensus that plant mitochondria and the α purple bacteria are very closely related. That Rhodospirillum and the plant mitochondria are so similar and that the mitochondria of animals and fungi are more distant could provide an explanation for the presence of the H^+-PPase in the first two groups and its absence from the latter two.

A notable exception to the otherwise strong link between plant mitochondria and the α purple bacteria is the unicellular green alga Chlamydomonas reinhardtii. The mitochondrial genome of C. reinhardtii is quite different from other plants both in its genomic structure and rRNA sequences (Gray, 1988; Van de Peer, 1990). The small size of the mitochondrial genome (16 kb) more resembles that of animals than plants, where the smallest genome is an order of magnitude larger. It is also devoid of introns and its mRNAs lack 5′ non-coding sequences - a characteristic of animal mitochondrial genomes (Gray, 1988). Analysis of its rRNA sequences places C. reinhardtii in a separate branch of the mitochondrial phylo-

genetic tree, prompting the suggestion that a second eukaryotic endosymbiosis could have occured within the plant line after Chlamydomonas separated (Gray, 1988).

Examination of C. reinhardtii and other related organisms for the presence of the H^+-PPase gene should provide interesting insights into the phylogeny of this enzyme and even perhaps provide some additional evidence relating to the question of monophyletic or polyphyletic origin of mitochondria.

The schemes presented above, describing the possible origins of the H^+-PPase in plants, remain highly speculative. Even though the hypothesis of the endosymbiotic origin of the H^+-PPase is not yet substantiated, it identifies those key organisms which could yield the answers to the phylogenetic origins of this enzyme. The presence of a H^+-PPase in archaebacteria, for instance, would argue against an endosymbiotic origin for the enzyme, whereas its absence in cyanobacteria would suggest that chloroplasts were an unlikely route. If the H^+-PPase is not found in C. reinhardtii, the mitochondrial hypothesis would become more credible and the enzyme could even play a role in determining mitochondrial phylogeny.

5.3. CONCLUSIONS

The availability of a H^+-PPase clone from Arabidopsis opens several avenues of research into the origins of this

enzyme as well as into its physiological role in plants.

Rapid screening of a large array of organisms for the presence of the gene both in functional and vestigial forms will allow determination of the phylogenetic origins of the H^+-PPase. In addition, regulation of the expression of its gene, whether translational or transcriptional, can be determined by estimation of mRNA levels in tissues with different H^+-PPase activity levels. The importance of the enzyme to the plant's metabolism could be assessed through inactivation of the gene either by site-directed mutagenesis or by expression of antisense mRNAs in transgenic plants.

The possibility that the enzyme translocates potassium ions in parallel with protons is presently investigated in several laboratories. If confirmed, it would allow exploration of the mechanisms of turgor regulation, stomatal guard cell swelling and leaf movement. The fact that requirement of K^+ for activity is specific to the plant H^+-PPase means that the enzyme may have undergone a functional modification from its more primitive counterpart in photosynthetic bacteria. It will be very interesting to compare the two sequences to determine how this adaptation has come about.

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

To my knowledge, there had been no reports previous to Sarafian & Poole (1989 - CHAPTER 2) which provided an unambiguous purification procedure for any plant vacuolar H^+-PPase. This study contributed the first size estimate (from SDS/PAGE) of the hydrolytic subunit of this enzyme in Beta vulgaris. Within months, our results were confirmed by Britten et al. (1989) in Beta and Maeshima & Yoshida (1989) in Vigna.

Functional-size estimates for H^+ translocation of the vacuolar H^+-PPase and the H^+-ATPase (Sarafian et al., 1992b - CHAPTER 3) are the first to be reported for any organism. Functional-size estimates of the H^+-PPase for substrate hydrolysis have been reported before (Chanson & Pilet, 1989) but this is the first refereed publication providing sufficient precision to suggest that one enzyme subunit is sufficient for the hydrolytic function. This is also the first report of functional sizes for hydrolysis and H^+ pumping of both the H^+-PPase and the H^+-ATPase in Beta.

I was the first to isolate, clone and sequence a cDNA of the H^+-PPase gene (CHAPTER 4). This was also the first conclusive evidence, based on sequence analysis, that the H^+-PPase belongs to a novel group of proton pumps and that it is not related to other inorganic soluble PPases.