# Characterization of the Vacuolar $H^+$-ATPase of Higher Plants

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Dedicated to Petra, without whose love
and support I would never have survived

## Abstract

The tonoplast $H^+$-translocating ATPase of *Beta vulgaris* L. was partially purified by Triton X-100 solubilization and Sepharose 4B chromatography resulting in the enrichment of two polypeptides (57 and 67 kDa). Kinetic analysis of [$\alpha$-$^{32}$P]BzATP labeling identified the 57 kDa polypeptide as a nucleotide-binding subunit with a possible regulatory function. In addition, [$^{14}$C]DCCD-labeling identified a 16 kDa polypeptide as a putative transmembrane proton channel. It is concluded that the tonoplast $H^+$-ATPase is a multimer composed of at least three polypeptides.

Anti-57 and anti-67 kDa sera reacted with polypeptides of the corresponding size in bovine chromaffin granules, bovine clathrin-coated vesicles, and yeast vacuolar membranes, suggesting common structural features and common ancestry for endomembrane $H^+$-ATPases of different organelles and different phyla. Anti-57 serum was used to isolate a cDNA encoding the corresponding subunit from *Arabidopsis*. Protein sequence analysis revealed homologies between endomembrane, $F_OF_1$ and archaebacterial ATPases, suggesting that these different classes of ATPases have evolved from a common ancestor.

THE TRANSLATION OF THE AUTHOR'S ABSTRACT BY MARIO FILION AND FRANCIS OUELLETTE, PAGE ii, WAS NOT MICROFILMED. PLEASE REFER, IF NEED BE, TO THE ORIGINAL THESIS. DEPOSITED WITH THE UNIVERSITY CONFERRING THE DEGREE.

LA TRADUCTION DU RESUME ANALYTIQUE DE L'AUTEUR PAR MARIO FILION ET FRANCIS OUELLETTE, PAGE ii, N'A PAS ETE MICROFILME. VEUILLEZ VOUS REFERER AU BESOIN A LA THESE ORIGINALE DEPOSEE A L'UNIVERSITE QUI A CONFERE LE GRADE.

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## CHAPTER 2. MATERIALS AND METHODS

## Acknowledgements

The diversity of techniques employed in this thesis reflects the generous and supportive nature of my colleagues. Although the list of people I am indebted to seems as long as the thesis itself, actions above and beyond the call of friendship necessitate the mention of a few. I would like to thank Philip A. Rea for his valuable contributions to the purification of the enzyme and Stephen K. Randall for his help with *in vitro* translation. I am grateful to Vahé Sarafian and Karen Ketchum for assisting in the drudgery of membrane purifications. If it were not for the guarantee from Mario Filion and Francis Ouellette (those bums) that every experiment in molecular biology takes less than an hour and that I would only need one clone, I would have never started that project. If it were not for their constant advice, protocols, chemicals and reminders of the aforementioned guarantee, I never would have finished. David (the shredder) Brummell must be thanked for his critical (if not brutal) proof reading of this thesis. I would especially like to thank Ronald J. Poole for assuming the most helpful role a supervisor can, that of a good friend. To Petra Manolson-Kühl I owe thanks for valuable technical assistance, advice and emotional support. It is really the friendship of all the above mentioned people for which I am truly thankful. I am sorry to leave for fear of losing it.

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## Preface

This thesis has been assembled in accordance with the regulations of the Faculty of Graduate Studies and Research of McGill University. It consists of a Literature Review (Chapter 1), Results in the form of three manuscripts suitable for publication (Chapter 3, 4 and 5) and a General Discussion (Chapter 6). To minimize iteration, all the Materials and Methods have been consolidated into Chapter 2 and References placed after Chapter 6.

Chapter 3 has been published by Manolson, M. F., Rea, P. A. & Poole, R. J. (1985), J. Biol. Chem. 260 (22), 12273-12279. Minor revisions have been made to the text in order to conform to McGill's "Guidelines Concerning Thesis Preparation". Philip A. Rea was responsible for the solubilization, phospholipid activation, and chromatography of the enzyme resulting in Figures 3.5, 3.6, and 3.7. All other work was done by the author.

Chapter 4 has been published by Manolson, M. F., Percy, J. M., Apps, D. K., Xie, X., Stone, D. K., Poole, R. J., (1986) In Membrane Proteins: Proceedings of the Membrane Protein Symposium, San Diego, California, Eds. Steven C. Goheen, Published by Bio-Rad, 427-434. Judith M.Percy and David K. Apps isolated the chromaffin granule membranes, Xiao-Song Xie and Dennis K. Stone isolated the clathrin coated vesicles, Michael Harrison and David J. Clark isolated plasma membranes

from *Clostridium pasteurianum*, Carl Yamashiro and Tom H. Stevens isolated the yeast vacuolar membranes, Richard Humbert and Robert D. Simoni purified the bacterial $F_1$ ATPase, Richard E. McCarty purified the chloroplast $F_1$ ATPase, and Sharon Ackerman and Peter Coleman purified the mitochondrial $F_1$ ATPase. All other work was done by the author.

Chapter 5 is a manuscript in preparation, which except for the phylogenetic comparisons (section 5.2.7) will be submitted to J. Biol. Chem. The phylogenetic comparisons will be submitted separately as part of a collaborative work with Lincoln Taiz, Emma Jean Bowman, Barry Bowman and Masasuke Yoshida. An Introduction for Chapter 5 was redundant with Chapter 1 and was thus omitted. Oligonucleotides were synthesized by Ken Deugau (Queen's University) and Claude Lemieux (Laval University). *In vitro* translation was performed by Stephen K. Randall. All other work was done by the author.

| | |
|---|---|
| BSA | bovine serum albumin |
| BzATP | 3-O-(4-benzoyl)benzoyladenosine 5'-triphosphate |
| DCCD | N,N'-dicyclohexylcarbodiimide |
| $ddH_2O$ | deionized and distilled water |
| DES | diethylstilbestrol |
| DIDS | 4,4'-diisothiocyano-2,2'stilbene disulfonic acid |
| DMSO | dimethyl sulfoxide |
| DTT | dithiothreitol |
| EDTA | ethylene diamine tetraacetic acid |
| EGTA | ethylene glycol-bis ($\beta$-aminoethyl ether) N,N,N',N'-tetraacetic acid |
| IPTG | isopropyl $\beta$-D-thiogalactopyranoside |
| Mes | 2-(N-morpholino)ethanesulfonic acid |
| NBD-Cl | 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole |
| NEM | N-ethylmaleimide |
| PAF | platelet activating factor |
| PAGE | polyacrylamide gel electrophoresis |
| PIPES | piperazine-N,N'-bis(2-ethanesulfonic acid) |
| PMSF | phenylmethylsulfonylfluoride |
| SDS | sodium dodecyl sulfate |
| SM | 10 mM Tris-Cl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$ and 0.01% gelatin |
| TCA | trichloroacetic acid |
| Tris | tris(hydroxymethyl)aminomethane |
| TSB | Tris saline buffer (10 mM Tris-Cl pH 7.3 and 0.9% NaCl) |
| TTSB | 0.05% Tween 20 in TSB |

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# Chapter 1. LITERATURE REVIEW

## 1.1 INTRODUCTION

Élie Metchnikoff (1905) described a series of experiments performed in the late 19$^{th}$ century in which pH-sensitive dyes (blue litmus, alizarin sulpho-acid and neutral red) were used to demonstrate that the digestive vacuoles of unicellular organisms were acidic compartments. Metchnikoff hypothesized that an acidic medium was necessary for the digestive reactions of the "diastase". Since this time, other endomembrane organelles have been found to contain acidic interiors, and the list of functions attributed to the pH gradient is as long as the list of organelles themselves (see section 1.4.1). The enzyme responsible for the acidification has direct access to the cell's main energy source, ATP. It is the characterization of one such proton-translocating ATPase, responsible for plant vacuole acidification, which is the concern of this thesis. The characterization of this enzyme is important not only for an understanding of the formation and regulation of vacuolar acidification, but also to explore evolutionary relationships. The approximately 8000 papers published on cation-translocating ATPases between 1970 and 1978 (Pedersen *et al.*, 1982) described ATPases which could be categorized into two major groups, the P-type and the $F_OF_1$ ATPases (both of which will be reviewed briefly in sections 1.2 and 1.3 respectively).

Initial reports in the late 1970s suggested that ATPases associated with endomembrane organelles fit neither of the established groups. The common features of these enzymes argued rather for the existence of a third major group of cation-translocating ATPases. The evidence to date on these endomembrane ATPases is presented in section 1.4 including, for the sake of completion, that of the author. Comparison of $F_OF_1$ with endomembrane ATPases has led to evolutionary speculation on the origins of these enzymes. The possibilities have become even more far-reaching considering the new information on archaebacterial ATPases, which will be reviewed in section 1.5.

## 1.2 P-TYPE ATPases

The "P" in P-type ATPase stands for phosphorylated intermediate (Pedersen and Carafoli, 1987), the common feature amongst $H^+$-ATPases from the plasma membrane of plants (reviewed in Briskin and Poole, 1983), yeast (reviewed in Serrano, 1988), and *Neurospora* (reviewed in Goffeau and Slayman, 1981), as well as the $Na^+,K^+$-ATPase (reviewed in Cantley, 1981), the sarcoplasmic reticulum $Ca^{2+}$-ATPase (MacLennon *et al.*, 1986), the gastric $H^+,K^+$-ATPase (Schull and Lingrel, 1986), and the $K^+$-ATPase of *E. coli* (Hesse *et al.*, 1984) and *Streptococcus faecalis* (Furst and Solioz, 1986). During the reaction pathway of these enzymes, a covalent phosphorylated intermediate is formed when the $\gamma$ phosphate of ATP reacts with a single aspartic acid residue. The formation of this intermediate is

inhibited by vanadate which resembles the transitional state of the $\gamma$ phosphate. Thus vanadate-sensitivity is another common characteristic of this group. The more established alias of the P-type ATPases, the $E_1E_2$ ATPases, refers to the two major conformational states of the enzyme ($E_1$ and $E_2$) during the reaction pathway (reviewed by Cantley, 1981). As evidence suggests other groups of ATPases undergo conformational changes during their reaction cycles, it is now thought inappropriate to group ATPases by this characteristic (Pedersen and Carafoli, 1987). Plasma membrane ATPase is yet another alias referring to the common location of these enzymes, although it also seems inappropriate due to the number of exceptions. The $Ca^{2+}$-ATPase located on the sarcoplasmic reticulum is a P-type ATPase while the non-phosphorylated *E. coli* $F_0F_1$ $H^+$-ATPases and the archaebacterial $H^+$-ATPases are located on the plasma membrane (see section 1.5). The P-type ATPase of yeast has also been found in Golgi-derived vesicles, although the enzyme is presumably en route to the plasma membrane along the secretory pathway (Holcomb *et al.*, 1988). The P-type ATPases are similar in that they all consist of one approximately 100 kDa polypeptide which is thought to form six to eight membrane-spanning regions (Serrano, 1988) and which contains both catalytic site and ion channel. The $Na^+,K^+$-ATPase does have an additional 50 kDa polypeptide which is necessary for activity, although its function is unknown (Cantley, 1981).

The common structural and mechanistic features of this group suggest a common evolutionary origin. Analysis of nucleic acid and amino acid sequences has supported the notion

that all the ATPases with a phosphorylated intermediate evolved from a common ancestor (Serrano, 1988). Although P-type ATPases have similar functions to $F_OF_1$, endomembrane, and archaebacterial $H^+$-ATPases, Serrano (1988) suggests that they have evolved independently. There is however evidence to the contrary. It has been suggested (Garboczi *et al.*, 1987) that the phosphorylation region of the P-type ATPase (SDKTGTIT) is homologous to the ATP-binding site of the $\beta$ subunit of $F_OF_1$ ATPase from *E. coli* (TTKKGSIT). The significance of three identical amino acids and three conservative changes over an eight amino acid overlap is questionable. A computer search of the EMBL DNA sequence library with the nucleotide sequence of the 70 kDa polypeptide of the carrot vacuolar $H^+$-ATPase identified both $F_OF_1$ and P-type ATPases (Lincoln Taiz, personal communication) suggesting a relationship between the different classes of ATPases. This result conflicts with the results from computer searches done at an amino acid level which show no homology between P-type and the $F_OF_1$ and endomembrane ATPases (Lincoln Taiz, Barry and Emma Jean Bowman, personal communications, and this thesis).

## 1.3 $F_0F_1$ ATPases

The name $F_0F_1$ symbolizes the division of labour within this enzyme between proton conductance ($F_0$) and ATP hydrolysis or synthesis ($F_1$). When mitochondrial or chloroplast inner membranes are treated with chelating agents in low ionic strength buffers, oxidative or photosynthetic electron transport proceeds at very high rates relieved of its customary responsibility for ATP synthesis. The first component found which could recombine electron transport with ATP synthesis was termed coupling factor 1 or $F_1$ (McCarty, 1985). The "O" in $F_0$ stands for oligomycin (Efraim Racker, personal communication), an antibiotic which inhibits animal but not bacterial $F_0F_1$ ATPase activity through interaction with one of the $F_0$ subunits (Perlin *et al.*, 1985). The most universal indicator of $F_0F_1$ ATPase activity is sensitivity to azide, although the mode of azide inhibition is still unknown (Noumi *et al.*, 1987). The common alias, ATP synthetase (or ATP synthase) points out the direction in which these $H^+$-ATPases commonly, although not always, function. These enzymes are found in the inner plasma membrane of respiring or photosynthesizing bacteria (reviewed in Downie *et al.*, 1979; Dunn and Heppel, 1981; Fillingame, 1981), the inner membrane of mitochondria (reviewed in Catterall and Pedersen, 1973; Criddle *et al.*, 1979) and the thylakoid membrane of chloroplasts (reviewed in McCarty, 1982), where they convert an electrochemical potential difference for protons into the chemical bond energy of ATP. In the case of

anaerobic bacteria such as *Streptococcus faecalis* (Kobayashi *et al.*, 1982) and *Clostridium pasteurianum* (Clark *et al.*, 1979) which have no electron transport chains, the ATPases are thought to be responsible for the generation of the proton gradient required for nutrient accumulation and other functions.

The $F_1$ component, once detached from the membrane-bound $F_0$ component by mild sonication or low ionic strength buffers and chelating agents, becomes a water-soluble enzyme capable of ATP hydrolysis but not synthesis. $F_1$ is made up of 5 types of subunits, $\alpha$ (53 to 55 kDa), $\beta$ (50 to 54 kDa), $\gamma$ (31 to 37 kDa), $\delta$ (17 to 20 kDa) and $\epsilon$ (8 to 14 kDa) with a proposed stoichiometry of 3:3:1:1:1. Studies have shown both the $\alpha$ and $\beta$ subunits to contain nucleotide-binding sites (reviewed in Futai and Kanazawa, 1983). The $\beta$ subunit is thought to contain the catalytic site as it reacts with catalytic-site specific inhibitors. The role of the $\alpha$ subunit is still unclear: it has been proposed to contain the catalytic site (Matsuoka *et al.*, 1982), to contribute to a catalytic site at its interface with the $\beta$ subunit (Williams and Coleman, 1982) or to have only a regulatory function (Dunn, 1980). In *E. coli*, only the $\alpha$, $\beta$ and $\gamma$ subunits are needed to hydrolyse ATP (Futai, 1977) but this complex is unable to bind to the $F_0$ component without the addition of the $\delta$ and $\epsilon$ subunits (Sternweis and Smith, 1977; Sternweis, 1978). This suggests that while $\gamma$ is involved in the hydrolysis, $\delta$ and $\epsilon$ function in binding the $F_0$ component to the $F_1$ component. Only three polypeptides (65.5, 57.5 and 43 kDa) have been identified in *Clostridium pasteurianum* $F_1$ ATPase

suggesting that this obligately anaerobic bacteria has no equivalent to the $\delta$ and $\epsilon$ subunits. (Clark *et al.*, 1979)

Sequence comparisons (Walker *et al.*, 1985) show conservation of $\alpha$, $\beta$ and $\gamma$ subunits between bacterial, chloroplast, and mitochondrial ATPases, with immunological evidence (Rott and Nelson, 1981) and sequence homology showing the $\beta$ subunit as the most conserved. There is partial sequence homology between the $\alpha$ and $\beta$ subunits suggesting that these two polypeptides may have originated from one common ancestral gene (Walker *et al.*, 1982a). There is very little homology among species for the $\gamma$ and $\epsilon$ subunits. The highest homology reported for either subunit is 20% identity for the $\epsilon$ subunit in *E. coli* and chloroplast (Futai and Kanazawa, 1983), both of which are weakly homologous to the mitochondrial $\delta$ subunit (Walker *et al.*, 1982b).

The $F_O$ component has been shown to form a transmembrane proton channel (reviewed in Hoppe and Sebald, 1984; Schneider and Altendorf, 1987). Bacterial $F_O$ has three subunits (a,b,c), chloroplast $F_O$ has four subunits (I, II, III, IV), mitochondrial $F_O$ has five subunits (su 6, su 8, su 9, OSCP and $F_6$) and *Clostridium pasteurianum* $F_O$ has only one identified subunit ($f_a$). Subunit correspondence between organisms is still an issue of debate except for the dicyclohexyl-carbodiimide- (DCCD-) binding subunits (subunit c in bacteria, subunit III in chloroplast, subunit 9 in mitochondria and subunit $f_a$ in *Clostridium pasteurianum*) which all show cross-species homology (Sebald and Hoppe, 1981). The isolated DCCD-binding subunit (also called proteolipid) in mitochondria and

chloroplasts has been reported to be able to form a DCCD-sensitive proton channel by itself (Konishi *et al.*, 1979; Sigrist-Nelson, 1980). Stoichiometry for the proteolipid ranges from 6 to 12 copies with each subunit capable of forming two membrane-spanning α-helices. The hydrophobic inhibitor DCCD binds covalently to an acidic residue (aspartic acid in *E. coli*, glutamic acid in mitochondria and chloroplasts) located in the middle of a hydrophobic stretch of the proteolipid, perhaps inhibiting by physically blocking the proton channel (reviewed in Azzi *et al.*, 1984). The $F_OF_1$ inhibitors, oligomycin, venturicidin and trialkyltin are also thought to function through interaction with the proteolipid (Linnett and Beechey, 1979; Perlin *et al.*, 1985).

Despite the differences in inhibitor sensitivities and subunit structure, there is no doubt as to a common origin of the $F_OF_1$ ATPases. The subunits involved in the primary functions of ATP hydrolysis (α, β and γ ) and proton conductance (DCCD-binding protein) are conserved. Further evidence comes from the endosymbiotic theory which suggests that the organelles on which these enzymes reside also have a common origin. Mitochondria and chloroplasts are semi-autonomous organelles containing prokaryotic-like genes and protein-synthesizing machinery. On this evidence and from sequence homologies of rRNA, tRNA, ferredoxins and cytochromes it is suggested that mitochondria and chloroplasts evolved from prokaryotes (Dayhoff and Schwartz, 1981; Schwartz and Dayhoff, 1981). Although the primary function of ATP synthesis is the same for almost all $F_OF_1$ ATPases, their regulation must differ.

For instance, while the bacterial ATPases alternate between ATP synthesis and hydrolysis to accommodate aerobic and anaerobic conditions, chloroplast ATPases are regulated according to illumination, with ATP hydrolysis being inhibited in the dark. Perhaps the differences in regulation have resulted in the differences in inhibitor sensitivities and in the minor subunits. It has been speculated that the simpler subunit composition of *Clostridium pasteurianum* reflects the earlier evolutionary status of an obligate anaerobe (Maloney and Wilson, 1985).

## 1.4 ENDOMEMBRANE ATPases

### 1.4.1 Location and Role of Endomembrane ATPases

The aliases of this group (V-type ATPase or vacuolar ATPase (reviewed in Bowman and Bowman, 1986; Pedersen and Carafoli, 1987a; Schneider, 1987), acidic organelle ATPase (reviewed in Rudnick, 1986), $T_p$-type or tonoplast-type ATPase (reviewed in Sze, 1985), and microsomal $H^+$-ATPase (reviewed in Al-Awquati, 1986)), refer to the locations of these $H^+$-ATPases (summarized in Table 1.1). With the exception of the turtle urinary bladder luminal membranes (Gluck *et al.*, 1982) and the renal medulla microsomes (Gluck and Al-Awqati, 1984), these ATPases are located on intracellular organelles which require acidic interiors. Endomembrane ATPases hydrolyze ATP to ADP and $P_i$, using the energy of the chemical bond to pump protons

**Table 1 1 Locations of endomembrane ATPases**

| Organelle | Source | Reference |
|---|---|---|
| CHROMAFFIN GRANULES | Bovine Adrenal Medullae | Apps and Schatz, 1979<br>Cidon and Nelson, 1983<br>Dean *et al.*, 1986 |
| CLATHRIN-COATED VESICLES | Bovine Brains | Stone *et al.*, 1982<br>Forgac *et al.*, 1983 |
| ENDOPLASMIC RETICULUM | Rat Liver | Rees-Jones and Al-Awqati, 1984 |
| ENDOSOMES | Rat Liver | Saermark *et al*, 1985 |
| GOLGI | Corn Coleoptiles | Chanson *et al*, 1984 |
| | Rat Liver | Glickman *et al*, 1983 |
| | Sycamore (cell culture) | Ali and Akazawa, 1986 |
| LUMINAL URINARY BLADDER | Turtle | Gluck *et al*, 1982 |
| LYSOSOMES | Baby Hamster Kidney (Cell Suspension) | Galloway *et al*, 1983 |
| | Rat Liver | Ohkuma *et al*, 1981<br>Schneider, 1981 |
| MULTIVESICULAR BODIES | Rat liver | Van Dyke, 1986 |
| PLATELET DENSE GRANULES | Pig Liver | Dean *et al*, 1984 |
| RENAL MEDULLA MICROSOMES | Bovine | Gluck and Al-Awqati, 1984 |
| | Rat | Kaunitz *et al*, 1985 |
| SYNAPTOSOMES | Rat Forebrain | Cidon *et al*, 1983 |
| VACUOLE | CAM Plant | Jochem and Luttge, 1987 |
| | Corn Coleoptiles | Mandala and Taiz, 1985a |
| | Oat Roots | Wang and Sze, 1985 |
| | Red Beet | Poole *et al.*, 1984<br>Bennet *et al*, 1984a |
| | Radish | Michelis *et al*, 1983 |
| | Sugarcane | Thom *et al*, 1983 |
| | *Neurospora crassa* | Bowman and Bowman, 1982 |
| | *Saccharomyces carlsbergensis* | Lichko and Okorokov, 1984 |
| | *Saccharomyces cerevisiae* | Kakinuma *et al*, 1981 |

into the interiors of their respective organelles. The resulting proton gradient is used as the driving force for secondary transport, or to provide an acidic pH for optimal activity of degradative enzymes, protein processing, receptor-ligand dissociation or urinary acidification (detailed in Table 1.2). Rausch *et al.* (1987) have demonstrated the direct coupling of secondary transport to the proton gradient generated by the tonoplast $H^+$-ATPase by showing that antibodies specific to the tonoplast ATPase also inhibited active glucose transport into tonoplast vesicles.

### 1.4.2 Inhibitors of Endomembrane ATPases

The first evidence that endomembrane ATPases should be considered as a new class was their insensitivity to $F_OF_1$-type inhibitors (azide, oligomycin and efrapeptin) and to P-type inhibitors (vanadate and ouabain). The common indicators of endomembrane ATPases are sensitivity to N-ethylmaleimide (NEM), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), DCCD and nitrate. Although NEM, NBD-Cl and DCCD also inhibit the other classes of ATPases, the low effective concentrations ($\mu$M range) of NEM and NBD-CL and the high concentration range of DCCD (mM range) distinguish the endomembrane-type.

Inhibition by the alkylating adenine-analog, NBD-Cl, has been shown to be protectable by nucleotides (Dean *et al.*, 1984; Wang and Sze, 1985; Mandala and Taiz, 1986; Bowman *et al.*, 1986; Arai *et al.*, 1987b; Moriyama and Nelson, 1987a), to follow pseudo first-order kinetics (Randall and Sze, 1987; Uchida *et al.*, 1988), and to be reversible by either DTT

Table 1.2 Function of proton gradient

| Organelle | Function |
|---|---|
| CHROMAFFIN GRANULES | Energy source for catecholamine accumulation (Bashford *et al*., 1976) |
| CLATHRIN-COATED VESICLES AND ENDOSOMES | Acidification triggers receptor-ligand dissociation (reviewed in Mellman *et al* , 1986) |
| ENDOPLASMIC RETICULUM | *Translocation of proteins and sugar (Rees-Jones and Al-awqati, 1984) |
| GOLGI | *Acidic interior required for processing of newly synthesized proteins (Zang and Schneider, 1983) |
| LUMINAL URINARY BLADDER | Protons pumped into the bladder lumen result in urinary acidification (Gluck *et al* , 1982) |
| LYSOSOMES | Acidic interior required for optimal activity of lysosomal degradative enzymes (Coffey and de Duve, 1968) |
| PLATELET DENSE GRANULES | Energy source for serotonin accumulation, (Dean *et al* , 1984) |
| RENAL MEDULLA MICROSOMES | Urinary acidification in mammalian collecting duct, (Gluck and Al-Awqati, 1984) |
| SYNAPTOSOMES | Energy source for accumulation of neurotransmitters, (Anderson *et al* , 1982) |
| VACUOLE | Energy source for metabolite accumulation, acidic interior required for optimal activity of degradative enzymes, regulation of cytoplasmic pH (reviewed in Boller and Wiemken,1986) |

*speculation

(Randall and Sze, 1987) or β-mercaptoethanol (Uchida *et al.*, 1988). These results suggest that NBD-Cl interacts with either a tyrosine or cysteine located in the catalytic site. Uchida *et al.* (1988) also showed NBD-Cl inhibition to be pH-dependent with optimal inhibition in alkaline medium, suggesting that at least in yeast the modified residue is tyrosine. ATP-protectable labeling with [$^{14}$C]-NBD-Cl was found only on one polypeptide (Bowman *et al.*, 1986; Mandala and Taiz, 1986; Aria *et al.*, 1987; Randall and Sze, 1987; Uchida *et al.*, 1988) identifying it as the catalytic subunit (see section 1.4.6)

Irreversible inhibition of the endomembrane ATPases by the sulfhydryl reagent, NEM, can only be partially protected by nucleotides (Bowman *et al.*, 1986; Griffith *et al.*, 1986; Percy and Apps, 1986; Arai *et al.*, 1987b; Cuppoletti *et al.*, 1987; Moriyama and Nelson, 1987a) suggesting that NEM is interacting at several sites, one of which is a nucleotide-binding site containing cysteine. Randall and Sze (1987) showed that NEM inhibited plant tonoplast $H^+$-ATPase activity in a non-linear fashion, again suggesting that NEM modifies several sites. These complex kinetics concur with the ATP-protectable [$^{14}$C]-NEM labeling of two nucleotide-binding subunits in plant tonoplast $H^+$-ATPases (Stephen K. Randall, unpublished results) and three subunits in chromaffin granule $H^+$-ATPase (Moriyama and Nelson, 1987a). Sequencing of the plant catalytic subunit has confirmed the presence of cysteine residues within the putative catalytic region of the polypeptide (Lincoln Taiz, personal communication).

With the exception of plant Golgi $H^+$-ATPases (Chanson and Taiz, 1985; Ali and Akazawa, 1986) endomembrane ATPases are inhibited by nitrate. Nitrate and other chaotropic monovalent ions ($SCN^-$ and $ClO_4^-$) were shown to be competitive inhibitors of plant tonoplast $H^+$-ATPases (Griffith *et al.*, 1986). Lack of positive or negative cooperative interaction between nitrate and ATP (also a competitive inhibitor) with respect to enzyme inhibition led Griffith *et al.* (1986) to conclude that nitrate inhibition was not due to its stereochemical similarity to the γ phosphate of ATP, but rather due to its chaotropic properties. Irreversible inhibition by higher concentrations of chaotropic anions was shown to be caused by the release of the peripherally bound nucleotide-binding subunits (Rea *et al.*, 1987a).

DCCD, a hydrophobic carboxyl reagent, not only inhibits all the endomembrane ATPases, it has also been shown to inhibit facilitated proton diffusion through the reconstituted 17 kDa polypeptide from clathrin-coated vesicle ATPase (Sun *et al.*, 1987). This suggests that the site of DCCD interaction is the proton channel-forming subunit.

The anion-channel blockers, diethylstilbestrol (DES) and 4,4'-diisothiocyano-2,2'stilbene disulfonic acid (DIDS) are irreversible inhibitors of endomembrane ATPases from plant vacuoles (Thom *et al.*, 1983; Aoki and Nishida, 1984; Poole *et al.*, 1984; Mandala and Taiz, 1985a; Marin *et al.*, 1985), plant Golgi (Chanson and Taiz, 1985) and bovine renal medulla (Gluck and Caldwell, 1987). For the plant vacuolar $H^+$-ATPase, DIDS inhibition could be protected by $Cl^-$ suggesting that DIDS and

$Cl^-$ bind to the same site(s) on the enzyme (Churchill and Sze, 1984).

### 1.4.3 Endomembrane ATPases are Electrogenic Proton Pumps

MgATP-generated formation of inside-positive membrane potentials and inside-acid pH gradients in membrane vesicles, as well as in endomembrane organelles *in situ*, have been attributed to endomembrane ATPase activity through similarities in kinetic parameters, substrate specificities and inhibitor sensitivities. Formation of membrane potentials and pH gradients is sensitive to NBD-Cl, NEM, nitrate, DCCD, DIDS, and DES but insensitive to vanadate, ouabain, azide and oligomycin (Ohkuma *et al.*, 1982; Cidon *et al.*, 1983; Bennett *et al.*, 1984; Gluck and Al-Awqati, 1984; Lichko and Okorokov, 1984; Poole *et al.*, 1984; Kaunitz *et al.*, 1985; Mandala and Taiz, 1985b; Saermark *et al.*, 1985; Wang and Sze, 1985; Xie and Stone, 1986; Moriyama and Nelson, 1987b). Churchill and Sze (1984) calculated the apparent $K_m$ for MgATP of proton pumping in plant vacuoles to be 0.1 mM, which is close to the 0.25 mM calculated for ATP hydrolysis in the same tissue (Wang and Sze, 1985). Poole *et al.* (1984) demonstrated similar pH dependency and substrate specificities for ATP hydrolysis and proton pumping.

### 1.4.4 Chloride Stimulation

With the exception of lysosomal $H^+$-ATPases (Cidon *et al.*, 1983; Moriyama *et al.*, 1986), endomembrane ATPase activity is stimulated by chloride. Churchill and Sze (1984) found the primary chloride effect to be an increase in $V_{max}$ for ATP

hydrolysis in plant vacuolar $H^+$-ATPases. In contrast, Griffith *et al.* (1986) found that in the presence of ionophores, chloride lowered the $K_m$ of the plant enzyme for MgATP while not affecting $V_{max}$. The different results suggest that when using sealed vesicles, the primary stimulating effect of chloride is to dissipate the electrical potential being built up in vesicles allowing a greater proton gradient to be generated. In the presence of ionophores there appears to be a direct effect of chloride on the enzyme. This has yet to be fully characterized although DIDS may be a useful probe to identify the putative chloride binding site (see section 1.4.3).

### 1.4.5 Substrate Specificity

Although all endomembrane ATPases show ATP as the most efficient substrate, there are considerable differences reported in the utilization of other nucleotides. The partially-purified enzyme from yeast can also hydrolyze (in decreasing order of preference) GTP, UTP, and CTP (Uchida *et al.*, 1985) while the partially-purified enzyme from plant vacuoles used also GTP and ITP (Mandala and Taiz, 1985a). In plant vacuoles (Poole *et al.*, 1984), chromaffin granules (Dean *et al.*, 1986), platelet dense granules (Dean *et al.*, 1984), and lysosomes (Ohkuma *et al.*, 1982), both ATP and GTP are substrates for proton-pumping activity. In contrast, a strict requirement for ATP was found for proton-pumping in endoplasmic reticulum (Rees-Jones and Al-Awqati, 1984), bovine kidney medulla (Gluck and Al-Awqati, 1984), clathrin-coated vesicles (Xie *et al.*, 1983) and Golgi from plant (Chanson and Taiz,

1985) or animal sources (Glickman *et al.*, 1983). Differences in nucleotide specificity may be due to ADP and nucleotide-5'-diphosphokinase in the membrane preparation (Xie *et al.*, 1983), non-specific phosphatase activity or impure sources of nucleotides.

### 1.4.6 Subunit Structure and Functional Molecular Mass

The endomembrane ATPases are multimeric enzymes consisting of at least three to possibly ten types of subunits (summarized in Table 1.3). The disparity between the numbers and molecular weights of the subunits from different sources reflect in part the different degrees of purification and different systems of electrophoresis. Evidence of structural and functional similarities of the nucleotide-binding subunits will be presented in chapters 3, 4, and 5. Note that in Table 1.3, all the preparations hydrolyze ATP, but those shown to be reconstitutively active in proton transport (Xie and Stone, 1986; Gluck and Caldwell, 1988) list the most subunits. Perhaps, as is the case with $F_0F_1$-ATPases, only three subunits are required for ATP hydrolysis, but ten subunits are required for coupling hydrolysis to proton pumping.

The most controversial subunit is the largest one (100 to 116 kDa) reported to be the catalytic subunit in chromaffin granules (Cidon and Nelson, 1986) although this was quickly refuted by the same group one year later (Moriyama and Nelson, 1987a). To explain the absence of the 115 kDa subunit in other preparations, Nathan Nelson (personal communication) stated that harsh denaturation (boiling samples in SDS buffer) caused

Table 1.3 Estimated Molecular Mass of Polypeptides

in Partially-Purified Preparations of Endomembrane $H^+$-ATPases

| Membrane: | Plant Tonoplast | | | Latex Lutoids | Fungal Tonoplast | | Clathrin-coated Vesicles | | Chromaffin Granules | | Renal Medulla Microsomes |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Source: | Beet | Corn | Oat | Rubber Plant | Neurospora | Yeast | Bovine Brain | | Bovine Adrenal Medulla | | Bovine Kidney |
| Reference: | 1 | 2 | 3,4 | 5 | 6 | 7,8 | 9[g] | 10 | 11,12 | 13,14 | 15[g] |
| Polypeptides: | | | | | kDa | | | | | | |
| | 67<br>57[a] | 72[b,(d)]<br>62[(d)] | 72[b,(e)]<br>60[(e)] | 66<br>54 | 70[b,e]<br>62 | 80[b,d]<br>64 | 116[(e)]<br>70[(e)]<br>58[(e)] | 100[b,e]<br>73[b,e]<br>58 | 70[b]<br>57 | 115[d,e,f]<br>72[d,e,f]<br>57 | 70<br>56 |
| | | | | | | | | | | | 45 |
| | | | | | | | 40 | 40 | 41 | | 42 |
| | | | | | | | 38 | 38 | | 39[d,e,f] | 38 |
| | | | | | | | 34 | 34 | | | |
| | | | | 23 | | | 33 | 33 | 33 | | 33 |
| | | | | | | | | | | | 31 |
| | 16[c] | 16[c] | 16[c] | 13 | 15[c] | 19.5[c] | 15[c] | 19 | 16[c] | 17[c] | 15 |
| | | | | | | | | 17 | | | |
| | | | | | | | | | | | 14 |
| | | | | | | | | | | | 12 |

1 Manolson et al., 1985
2 Mandala and Taiz, 1986
3 Randall and Sze, 1986
4 Randall and Sze, 1987
5 Marin et al., 1985
6 Bowman et al., 1986
7 Uchida et al., 1985
8 Uchida et al., 1988
9 Xie and Stone, 1986
10 Arai et al., 1987
11 Percy et al., 1985
12 Percy and Apps, 1986
13 Cidon and Nelson, 1986
14 Moriyama and Nelson, 1987a
15 Gluck and Caldwell, 1987

a: BzATP-binding
b: NBD-Cl-binding
c: DCCD-binding
d: 8-azido-ATP-binding
e: NEM-binding
f: ATP-binding
g: Reconstitutively active
(): Unpublished data

only the 115 kDa subunit to aggregate, thus preventing it from entering the gel matrix during electrophoresis. Perhaps harsh denaturation methods are required to dissociate the two other nucleotide-binding subunits (72 kDa and 39 kDa) reported by Moriyami and Nelson (1987) which together could possibly migrate to 115 kDa.

ATP-protectable binding of NBD-Cl, NEM and 8-azido-ATP to the second-largest subunit (66 to 80 kDa) and immunological evidence has led investigators to report this polypeptide as the catalytic subunit (Bowman *et al.*, 1986; Mandala and Taiz, 1986; Percy and Apps, 1986; Arai *et al* , 1987b; Moriyama and Nelson, 1987a). The most convincing evidence for identifying the catalytic site came from correlating the kinetics of inactivation by NBD-Cl to the kinetics of subunit labeling by [$^{14}$C]NBD-Cl (Randall and Sze, 1987; Uchida *et al.*, 1988). Sequence analysis of this subunit from plant and fungal sources revealed putative ATP-binding sites (Lincoln Taiz, Emma Jean Bowman and Barry Bowman, personal communications)

There are several unpublished reports (see Table 1.3) of a nucleotide-binding site on the third largest-polypeptide (54 to 64 kDa). Evidence of a possible regulatory function for this subunit is presented in Chapters 3 and 5.

The only other polypeptide with an assigned function is the DCCD-binding subunit (15 to 19.5 kDa) which, by analogy with $F_OF_1$ ATPases, was assumed to form the membrane-spanning proton channel. Purification in chloroform:methanol has shown this polypeptide to be very hydrophobic (Arai *et al.*, 1987a; Kaestner *et al.*, 1987; Rea *et al.*, 1987b) and to be able to

facilitate proton diffusion in a reconstituted system (Sun *et al.*, 1987). The hydrophobic character has been confirmed by sequence analysis of the 17 kDa subunit from chromaffin granules (Nathan Nelson, personal communication).

Calculations of the molecular mass of the functional endomembrane ATPases are summarized in Table 1.4. It is unclear whether the differences in reported mass reflect real differences in size between species, as the same method applied to the same source of enzyme gives different values when performed by different workers (Forgac and Berne, 1986; Xie and Stone, 1986).

Table 1.4. **Molecular mass of functional endomembrane ATPases**

| Mol. Mass (kDa) | Method | Source |
|---|---|---|
| 300-600 | estimated by gel filtration | plant vacuole (Randall and Sze, 1985) |
| 200 | estimated by gel filtration | latex lutoids (Marin *et al.*, 1985) |
| 400 | radiation inactivation | plant vacuole (Mandala and Taiz, 1985a) |
| 520 | radiation inactivation | *Neurospora* vacuole (Bowman *et al.*, 1986) |
| 530 | sedimentation equilibrium centrifugation | clathrin-coated vesicles (Xie and Stone, 1986) |
| 230 | sedimentation equilibrium centrifugation | clathrin-coated vesicles (Forgac and Berne, 1986) |
| 134 | sedimentation equilibrium centrifugation | chromaffin granule, (Dean *et al.*, 1987) |

## 1.5 ARCHAEBACTERIAL ATPase

Although their names and prokaryotic characteristics may suggest archaebacteria and eubacteria to be similar, phylogenetic data show these two groups to be as evolutionarily distant as eubacteria are from eukaryotes (see Chapter 6). Thus, differences between bacterial $F_0F_1$ ATPases and archaebacterial ATPases were expected. ATPases from the plasma membrane of *Halobacterium halobium* (Mukohata and Yoshida, 1987), *Halobacterium saccharovorum* (Hochstein *et al.*, 1987) *Sulfolobus acidocaldarius* (Wakagi and Oshima, 1985), and *Methanosarcina barkeri* (Inatomi, 1986), like endomembrane ATPase, are insensitive to $F_0F_1$-type and P-type inhibitors. They are not inhibited by azide, oligomycin, or low concentrations of DCCD, nor are they affected by vanadate or ouabain. Like the endomembrane ATPases, the ATPases from *S. acidocaldarius* (Konishi *et al.*, 1987; Lubben and Schafer, 1987) and *H. halobium* (Mukohata and Yoshida, 1987; Nanba and Mukohata, 1987) are inhibited by nitrate and the sulfhydryl reagents NBD-Cl and NEM. With the exception of *H. saccharovorum*, archaebacterial ATPases are activated by the anions sulfate and sulfite, although in contrast to endomembrane ATPases they are unaffected by chloride. Archaebacterial ATPases are similar to both endomembrane and $F_0F_1$-types in that they are multimeric and the catalytic portion can be removed from the membranes with EDTA in a low ionic strength buffer, implying it is peripherally bound.

Partial purification has revealed a subunit structure (summarized in Table 1.5) similar to that of the endomembrane ATPases, with two prominent polypeptides with a range of 86-62 and 64-49 kDa. As none of the purified ATPases in Table 1.5 have been shown to be reconstitutively active, the number of polypeptides may be underestimated. Although there is still no direct evidence as to function, similarities in inhibitor sensitivities, pH optima, and substrate specificities between the partially purified ATPase activities and ATP synthesis *in vivo* suggest an energy coupling function similar to that of the $F_OF_1$ ATPases. Antibodies raised against the partially purified ATPase from *H. halobium* cross-reacted with ATPase subunits from *S. acidocaldarius* and from plant vacuolar ATPases (Mokohata *et al.*, 1987). Further evidence of an evolutionary link between archaebacterial and endomembrane ATPases comes from sequence comparisons discussed in Chapters 5 and 6.

**Table 1.5 Estimated Molecular Mass of Polypeptides in Partially-Purified Preparations of Archaebacterial Plasma-Membrane-Associated ATPases**

| Source: | *Sulfolobus acidocaldarius* | | *Halobacterium halobium* | *Halobacterium saccarovorum* | *Methanosarcina barkeri* |
|---|---|---|---|---|---|
| Reference: | 1 | 2 | 3 | 4 | 5 |
| Polypeptides: (kDa) | 65<br>51 | 69<br>54 | 86<br>64<br>28 | 87<br>60 | 62<br>49<br>29<br>20 |
| Functional molecular mass: | | 360 | 300-320 | 350 | 420 |

1) Lübben and Schäfer, 1987
2) Konishi *et al.*, 1987
3) Mukohata and Yoshida, 1987
4) Hochstein *et al.*, 1987
5) Inatomi, 1986

# CHAPTER 2. MATERIALS AND METHODS

## 2.1 PROTEIN

### 2.1.1 Plant Material

Fresh red beet (*B. vulgaris* L.) storage roots with leaves intact were purchased commercially, stored at 4°C, and used within 1 week of purchase.

Columbia wild-type *Arabidopsis thaliana* seeds were a kind gift of Howard M. Goodman, Harvard Medical School. Seeds were sterilized in a 10% hypochlorite, 0.1% Triton X-100 solution and then washed with 15 x 10 ml sterile dd$H_2O$. Seeds were planted out on sterile 1.0% agar containing 4.3 g/l Murashige and Skoog salt mixture (Gibco) and 0.1 M sucrose (pH 5.8). Seedlings were raised in a growth chamber at 26°C under continuous lighting.

### 2.1.2 Isolation of Membrane Fractions and Mitochondria

Fractions enriched in tonoplast vesicles, plasma membrane vesicles, or mitochondria were isolated from red beet by sucrose density gradient centrifugation as described by Poole *et al.* (1984) except for the compositions of the homogenization and suspension media which were based on the recommendations of Scherer and Morré (1978). The homogenization medium consisted of 10 mM glycerophosphate, 0.65 M ethanolamine (adjusted to pH 8.0 with concentrated $H_2SO_4$), 0.28 M choline chloride, 26 mM

potassium metabisulfate, 2 mM salicylhydroxamic acid (Sigma), 0.2% (w/v) BSA (fraction V, essentially fatty acid-free, Sigma) 10% (w/v) insoluble PVP (Sigma), 5 mM dithiothreitol (DTT), 0.5 mM butylated hydroxytoluene (Sigma), 1 mM nupercaine (Sigma) and 1 mM phenylmethylsulfonyl fluoride (PMSF) buffered to pH 8.0 with 5 mM Tris-Mes. The suspension medium consisted of 1.1 M glycerol, 1 mM Tris-EDTA, 1 mM nupercaine, 0.5 mM butylated hydroxytoluene, 1 mM PMSF and 5 mM DTT buffered to pH 8.0 with 5 mM Tris-Mes. Choline and ethanolamine were included to minimize membrane degradation by phospholipase D, nupercaine to inhibit phospholipase A, butylated hydroxytoluene to inhibit lipid peroxidation, and glycerol or glycerophosphate to inhibit phosphatidic acid phosphatase activity.

Membrane vesicles from *Arabidopsis thaliana* were prepared using the same solutions and homogenization conditions as above except that the ratio of homogenization buffer (ml) to fresh tissue (g) was 4:1. The homogenate was centrifuged at 13,000 x g for 10 minutes (to remove mitochondria) and the supernatant centrifuged at 80,000 x g for 35 minutes. The microsomal pellet contained membrane vesicles from both tonoplast and plasma membrane and was used for Fig. 5.1 with no further purification.

### 2.1.3 Synthesis of BzATP

BzATP and [$\alpha$-$^{32}$P]BzATP were synthesized as described by Williams and Coleman (1982) and purified by paper chromatography. The purified compound migrated as a single spot upon TLC and had an RF identical to that reported by

Williams and Coleman. The specific activity of the [$\alpha$-$^{32}$P]BzATP used in these experiments was 40 mCi/mmol.

2.1.4 Labeling of Tonoplast Vesicles with [$\alpha$-$^{32}$P]BzATP

Photoirradiation was performed in the long-wavelength mode of a UVSL-58 Mineralight (Ultra-Violet Products, Inc.) at a sample-to-source distance of 3 cm. The samples for photoirradiation consisted of tonoplast (150 $\mu$g of membrane protein) in 200 $\mu$l of 30 mM Tris-Mes (pH 8.0) containing 0.3 mM $MgSO_4$, 50 mM KCl, and the concentrations of BzATP and ATP indicated in the text and legends. Photolysis was for 20 min in open, ice-cooled micro-Petri dishes of 1.5 cm inner diameter, after which time unbound label was removed by centrifuging the suspension through a Sephadex G-50 (fine) column. The samples were concentrated to 1 to 3 mg/ml protein by centrifugation for 45 min at 120,000 x g for solubilization and chromatography, or immediately prepared for SDS-PAGE.

2.1.5 Labeling of Tonoplast Vesicles with [$^{14}$C]DCCD

The membranes were labeled with [$^{14}$C]DCCD (60 mCi/mmol) by the incubation of tonoplast (250 $\mu$g/ml of membrane protein) in 30 $\mu$M [$^{14}$C]DCCD in 30 mM Tris-Mes, pH 8.0, for 25 min at room temperature. Unbound label was removed by centrifugation for 45 min at 120,000 x g, followed by resuspension of the pellet in ice-cold 30 mM Tris-Mes. The washing procedure was repeated at least twice. The membranes were prepared for solubilization and SDS-PAGE as described below.

### 2.1.6 Membrane Solubilization

Tonoplast vesicles were solubilized with Triton X-100 using the following procedure. The tonoplast suspension was adjusted to 2-4 mg/ml membrane protein with suspension medium, and an equal volume of Triton X-100 in 20% (w/v) glycerol, 5 mM Tris-Mes (pH 8.0), 1 mM Tris-EDTA and 5 mM DTT was added dropwise with constant stirring. The mixture was incubated on ice for 20 min and centrifuged at 200,000 x g for 35 min. The supernatant and pellet were collected separately. The tonoplast ATPase was routinely solubilized with a final concentration of 4% (w/v) Triton X-100.

### 2.1.7 Preparation of Phospholipids

The phospholipids used in these experiments were mixed soybean phospholipids (L-α-phosphatidylcholine, Type IV, from Sigma) containing approximately 40% phosphatidylcholine. For the phospholipid activation experiments, 25 mg phospholipid was dissolved in 2 ml chloroform in a screw-cap vial, and a thin film of phospholipid was formed on the sides of the vial by evaporation of the solvent under a stream of $N_2$. Residual solvent was removed by lyophilization for 3-4 h, and the vials were closed under $N_2$ and stored at -80°C. Immediately before use a 25 mg/ml stock suspension was prepared in 5 mM Tris-Mes (pH 8.0) and clarified by sonication for 20 min in a bath sonicator at 10°C.

### 2.1.8 Chromatography on Sepharose 4B

The solubilized ATPase was partially purified by gel

filtration on Sepharose 4B or CL-4B (Pharmacia). A 90 x 1 cm inner-diameter column packed with Sepharose 4B was equilibrated with running buffer (10% (w/v) glycerol, 0.3% (w/v) Triton X-100, 0.05 mg/ml phospholipid, 5 mM DTT, 1 mM Tris-EDTA, and 5 mM Tris-Mes, pH 8.0) then 2 to 3 mg of Triton X-100-solubilized tonoplast protein was applied. The column was operated at a flow rate of 3-4 ml/h at 5°C.

2.1.9 Preparation of Native Membranes, Triton X-100 Supernatants, and Sepharose 4B Fractions for SDS-PAGE

For the experiment shown in Fig. 3.4, a modification of the method of Piccioni *et al.*, (1982) was employed for the phase separation of phospholipid and Triton X-100 into diethyl ether and ethanol, respectively, before denaturation and SDS-PAGE of the chromatographic fractions. The removal of phospholipid and detergent before denaturation was found to be necessary in order to obtain high resolution in the low molecular size range of the polyacrylamide gels.

The samples were made 10% (w/v) with trichloroacetic acid, left on ice for 30 min, and centrifuged for 10 min in an Eppendorf microfuge. The supernatants were aspirated, the pellets were extracted with 1 ml of 90% (v/v) ethanol, and the centrifugation step repeated. The pellets from the ethanol extraction were dissolved in 50 µl of 3 mM Tris-Mes buffer (pH 8.0) containing 0.1% (w/v) SDS and extracted with 1 ml of ether for 10 min at room temperature. The two phases were resolved by centrifugation, the upper phase aspirated, and the aqueous phase extracted once more with ether before centrifugation and

aspiration of the upper phase. Residual ether was removed by evaporation under a stream of air. The final aqueous phase was frozen, lyophilized and denatured for 5 min at 100°C in denaturation buffer (5% (w/v) SDS, 5% (v/v) mercaptoethanol, 10 mM Tris-Mes, pH 8.0).

For the experiments shown in Figs. 3.3 and 3.8, the samples were treated as described above except that the ether extraction step was omitted.

The protein content of the denatured samples were determined by the method of Peterson (1977).

2.1.10 ATPase Assays

ATPase activity was measured either as the rate of ADP-dependent NADH oxidation in a coupled system containing phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase (Rea and Poole 1985) or as the rate of liberation of inorganic phosphate from ATP. Inorganic phosphate liberation was measured in a reaction volume of 0.3 ml containing 50 mM KCl, 3 mM Tris-ATP, 3 mM $MgSO_4$ and 40 mM Tris-Mes (pH 8.0). The reaction was initiated by the addition of membrane protein and allowed to proceed for 15-30 min at 37°C. The reaction was stopped and, if present, Triton X-100 and added phospholipid were precipitated by the addition of 0.3 ml of ice-cold 10% (w/v) trichloroacetic acid, 4% (v/v) perchloric acid. The samples were left on ice for 2 min, centrifuged for 3 min in an Eppendorf microfuge, and the supernatants assayed for $P_i$ by the method of Ames (1966).

## 2.2 IMMUNOLOGY

### 2.2.1 Antisera

Polyclonal antisera to the 57 and 67 kDa polypeptides of the beet tonoplast ATPase were raised as described by Vaitukaitis (1981) with modifications. Approximately 5.0 mg of partially-purified tonoplast $H^+$-ATPase was obtained from 6.3 kg of red beet roots using column chromatography as described in section 2.1.8. This material was subjected to SDS-PAGE, followed immediately by KCl staining (described below). Gel slices containing the visualized polypeptides were cut out, the gel matrix broken up by repeated passage through an 18 gauge needle and dialyzed against $ddH_2O$ to reduce the toxic concentrations of KCl and SDS. Lyophilization of the material resulted in a fine powder which was mixed with 1.5 volume of 10 mM Tris-Cl pH 7.3 and 0.9% NaCl (TSB) plus 1.5 volume of Freund's complete adjuvant (Sigma). This was repeatedly passed first through an 18 gauge needle followed by a 20 gauge needle until an emulsion was formed. TSB was added until the viscosity was such that the emulsion could easily pass through the 20 gauge needle otherwise the very difficult intradermal injections would become nearly impossible. Intradermal injections (10 to 15) were given over a wide area along the shaven backs of New Zealand White female rabbits in order to recruit many lymph nodes in the processing of antibodies. This was followed 21 days later by injecting the rabbits' backs with 6 subcutaneous boosters containing the same mix except for

having used Freund's incomplete adjuvant (Sigma) to form the emulsion. In total, approximately 50 μg antigen per rabbit had been injected. Two days after the boosters, blood was collected from an ear vein, allowed to clot overnight at 4°C and then centrifuged at 20,000 x g. The resulting sera were assayed for ATPase-specific antibodies through Western blotting. Rabbits injected with either the 57 or 67 kDa polypeptide tested positive for ATPase-specific antibodies and were sacrificed 7 days after the booster shots. Approximately 100 ml blood per rabbit was collected, which produced 70 ml serum.

Sera were stored at -70°C in small aliquots (1 ml) which were thawed as needed and then stored at 4°C for up to a month with no visible degradation. Repeated freeze-thawing cycles were avoided as this treatment resulted in precipitation of antibodies. Antibodies were partially purified by precipitation with ammonium sulfate. One volume whole serum and 1 volume 70% ammonium sulfate, 10 mM Tris-Cl, pH 7.5, 1 mM EDTA were mixed with gentle shaking for 5 hours at 25°C, and centrifuged at 10,000 x g for 15 min. The pellet was then washed in 35% ammonium sulfate, 10 mM Tris-Cl, pH 7.5, and 1 mM EDTA, centrifuged, resuspended in 0.5 volume TSB, and dialyzed against TSB overnight at 4°C. Partially-purified antibodies had a lower background on Western blots but their stability was subsequently reduced to two weeks at 4°C.

### 2.2.2 KCl Staining of SDS-Polyacrylamide Gels

KCl is used to precipitate SDS in the gel matrix leaving

regions of low SDS (i.e., high protein concentration) visible as clear bands. Following SDS-PAGE, the gel was incubated in 2 M KCl until it became opaque (1 to 2 minutes). To visualize the clear bands the gel was placed over a black background and illuminated from the side. The bands were quickly cut out as they were only visible for approximately 30 seconds. To stain afterwards with Coomassie Blue the KCl must first be washed out by a 30 minute incubation in 10% acetic acid.

### 2.2.3 Western Blotting and Immunodetection

During SDS-PAGE, 0.02% Pyronin Y (Sigma) was included in the protein sample buffer to serve as a dye front (running just behind Bromophenol Blue) that tranfers to nitrocellulose. Proteins were transferred from large polyacrylamide gels (greater than 15 by 15 cm) to nitrocellulose (Millipore, 0.45 $\mu$m) at 4°C at either 0.2 Amp overnight or 1 Amp for 3 hours in 20 mM Tris-Cl, pH 7.3, 150 mM glycine and 20% (v/v) methanol. Mini-polyacrylamide gels (less than 8 x 6 cm) were transferred in a mini-apparatus (Bio-Rad) at 100 V for 1 hour at 4°C. Following transfer, the blots were incubated in 2% BSA in TSB for either 1 hour at 37°C or overnight at 25°C in order to block all excess binding sites on the nitrocellulose. The blots were washed 3 x 10 minutes with 0.05% Tween 20 in TSB (TTSB) and incubated with the primary antibody (1:750 dilution with anti-57 sera, 1:1400 with anti-67 sera) for a minimum of 1 hour. The blots were again washed 3 x 10 minutes in TTSB and incubated in the secondary antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase, at a dilution of 1:1000

for a minimum of 1 hour. After 3 x 10 minute washes in TSB (Tween 20 inhibits peroxidase activity) the blots were incubated in 0.016% hydrogen peroxide and 0.5 mg/ml 4-chloro-1-naphthol for a maximum of 20 minutes. Once the immunoenzymatic staining was complete, the blots were washed twice in $ddH_2O$ and stored in the dark to prevent fading. If quantitation was required, $^{125}I$-protein A (approximately $10^6$ cpm/ml TTSB) was used instead of the secondary antibody followed by 6 x 10 minute washes in TSB. The blot was then wrapped in Saran Wrap (Dow Chemicals), exposed to X-ray film and the resulting autoradiogram was evaluated by densitometry.

2.2.4 Immunoprecipitation

*In vitro* translated, $^{35}S$-labeled proteins were first solubilized in 4% SDS in 50 mM Tris-Cl (pH 7.4) since the anti-57 antiserum only recognizes the SDS-denatured polypeptide. The SDS was diluted 10-fold with Triton such that the final buffer consisted of 0.4% SDS, 2% Triton, 150 mM NaCl, 4.8 mM EDTA, 50 mM Tris-Cl (pH 7.4) and 1 mM PMSF. To this mixture, anti-57 antiserum was added at a 1:750 dilution and the tubes incubated with a gentle rocking motion for 3 hours at 4°C. To 1 ml of the above mixture 100 $\mu$l of 20% Protein A-Sepharose (Pharmacia) was added and the incubation continued for a further 30 minutes. The antigen-antibody-protein A-Sepharose complex was then centrifuged down and washed extensively as described in Anderson and Blobel (1983). The immunoprecipitate was resuspended in electrophoresis denaturation buffer minus reducing agent (5% SDS, 10 mM Tris-

Cl, pH 8.0), boiled for 4 minutes, and the Protein A-Sepharose centrifuged out. Leaving the reducing agent out for this step minimized the amount of IgG (heavy chain) released, which otherwise distorts the gel pattern in the 50-60 kDa range. Immunoprecipitates were subjected to SDS-PAGE, and visualized through fluorography.

## 2.3 DNA/RNA

### 2.3.1 Screening λgt11 cDNA Library with Anti-57 kDa Antibodies

Antibodies specific to the 57 kDa polypeptide of the tonoplast $H^+$-ATPase were used to screen an *Arabidopsis thaliana* leaf cDNA λgt11 expression library (a kind gift of Howard M. Goodman, Harvard Medical School) by the method of Huynh (1985). *E. coli* Y1090 were infected with 50,000 plaque-forming units (pfu) and plated out on a 150 mm petri dish using 0.9% agarose as the top support medium. Agarose was used instead of agar to prevent sticking to the nitrocellulose during plaque lifts. Once the plaques had grown to a diameter of 1 mm, expression of the fusion protein was induced by overlaying with a Triton-free 0.45 μm nitrocellulose filter (Schleicher & Schuell, type BA85) saturated with 20 mM isopropyl β-D-thiogalactopyranoside (IPTG). After 3 hours of growth the filter was removed and a second nitrocellulose filter plaque replica was made from the same plate, the duplicate helping to identify false positives during immunodetection. As the antibodies seem only to recognize the SDS-denatured protein, after the plaque lifts the nitrocellulose filters were incubated in 0.1% SDS for 1 hour

followed by 3 x 5 minute washes in TTSB. Immunodetection was carried out using horseradish peroxidase as described in section 2.2.3. Putative positive clones were screened twice more to ascertain clonal purity.

### 2.3.2 Purification of λ DNA

Bacteriophage DNA was purified by immunoprecipitation of the phage particles. Briefly, phage plaques (200,000 pfu per 150 mm petri dish) were grown to confluence on solid support, overlaid with 12 ml of 10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$ and 0.01% gelatin (SM buffer) and shaken for 4 hours. The SM buffer was drawn off and centrifuged at 10,000 x g for 20 minutes to remove agarose and cellular debris. *S. aureus* cells conjugated to polyclonal antibodies directed against the λ particles (LambdaSorb$^{TM}$, Promega) were then used to immunoprecipitate the phage particles from the supernatant. Immunoprecipitation and subsequent DNA purification were done as described in the manufacturer's instructions.

### 2.3.3 Production of β-Galactosidase / 57 kDa Fusion Protein

To express the λgt11 fusion protein in large (μg) quantities, a recombinant lysogen in *E. coli* Y1089 was formed following the method of Huynh (1985). Y1089 cells were infected with the phage at a multiplicity of 5, plated out and incubated at 32°C. At 32°C the phage produce a temperature-sensitive repressor of the lytic cycle (cI857) which is inactive at 42°C. Single colonies were tested for temperature sensitivity at 42°C; colonies which grew at 32°C and not at

42°C were assumed to be lysogens. For expression of the fusion protein, a liquid culture of the recombinant lysogen was grown at 32°C until the late logarithmic stage was reached. The culture was quickly switched to 42°C and 10 mM IPTG added to the medium. When the bacteria had just begun to lyse, the cells were quickly harvested and resuspended in SDS protein denaturation buffer. The fusion proteins were identified by separating the crude lysate obtained above on SDS-PAGE, Western blotting, and immunodetection using Anti-$\beta$-galactosidase antibodies.

### 2.3.4 Subcloning of λgt11 Inserts into Bluescript

Partially EcoRI-digested λgt11 DNA was separated by agarose gel electrophoresis, and the insert DNA was eluted from the gel using Geneclean (BIO 101 Inc.) following the manufacturer's instructions. The insert was ligated to the dephosphorylated EcoRI-digested, KS M13$^+$ Bluescript vector (Stratagene Cloning Systems) as described by Maniatis *et al.* (1982). The ligated DNA was used to transform competent *E. coli* DH5αF' (Bethesda Research Laboratories) cells using $CaCl_2$ and heat shock as described by Hanahan (1985). Plasmid DNA was purified by the alkaline lysis minipreparation method of Maniatis *et al.* (1982) except that 2.5 M LiCl was used to precipitate RNA out of the preparation. The Bluescript plasmid containing the full length (1.88 kb) cDNA with its 5' end near Bluescript's T7 promoter will be referred to as p57kDa.

### 2.3.5 *In Vitro* Transcription from p57kDa

One μg of the p57kDa vector was used to *in vitro* transcribe 3 μg of RNA using the T7 promoter as described by Pelletier and Sonenberg (1984) with the modifications of Nielson and Shapiro (1986). Hind III digestion was used to define the endpoint of transcription 10 bp ~~up~~downstream from the 3' end of the insert DNA. Transcription was monitored by the incorporation of [$^3$H]-UDP (35 Ci/mmol). Unincorporated nucleotide was removed from the synthesized RNA using the G-50 spun-column method of Maniatis *et al.* (1982).

2.3.6 RNA and poly(A)$^+$ RNA Isolation

RNA was isolated from Columbia wild type *Arabidopsis thaliana* by extraction with guanidinium isothiocyanate as described by Lizardi (1983) and Chirgwin *et al.* (1979) with modifications. Entire plants were harvested after two weeks of growth, immediately frozen in liquid nitrogen, and then pulverized to a fine powder with mortar and pestle. One gram of material was resuspended in 7 ml homogenizing buffer (4.0 M guanidinium thiocyanate (ICN), 0.5% (w/v) sodium N-lauryl sarcosine (Sigma), 25 mM sodium citrate (pH 7.0) and 0.7% (v/v) β-mercaptoethanol) and mixed for 3 minutes using a Polytron at medium speed. The homogenate was centrifuged for 10 min at 10,000 x g at 10°C. The supernatant (3.5 ml) was then layered on top of a 3 ml cesium chloride cushion (5.66 M CsCl, 60 mM EDTA, pH 7.0) in a polyallomer tube, and centrifuged for 20 hours at 95,000 x g at 20°C. The resulting pellet was then washed in 70% ethanol, dried under vacuum and resuspended in dd$H_2O$. Poly(A)$^+$ RNA was obtained from total RNA by

chromatography on oligo (dT)-cellulose (Boehringer Mannheim Biochemicals) as outlined by Davis *et al.* (1986). For all the procedures dealing with RNA, all metal and glassware were baked at 400°C for a minimum of 3 hours. All solutions (except Tris) were treated for a minimum of 1 hour with 0.1% diethylpyrocarbonate (Sigma) prior to autoclaving. Solutions containing Tris were made with $ddH_2O$ pretreated with diethylpyrocarbonate.

### 2.3.7 DNA Sequencing

Single stranded DNA was sequenced by the dideoxynucleotide chain termination method of Sanger (1977). Single stranded template DNA was obtained by infecting *E. coli* DH5αF' containing the Bluescript plasmid with the helper phage M13K07 as described by Vernet *et al.* (1987). The sequencing reactions were carried out using the Sequenase™ kit (United States Biochemical Corporation) following the manufacturer's instructions. Primers used were either T7 or SK (Stratagene Cloning Systems) or were custom synthesized (see section 2.3.8). In regions with compressions, reactions were repeated using dITP instead of dGTP in the nucleotide mix. Sequence data were compiled on the program PCGENE (Intelligenetics). Both strands were sequenced to completion.

### 2.3.8 Oligonucleotide Synthesis and Purification.

For primer extension of DNA and RNA, the selection of oligonucleotides were done in accordance with Barnes's rules (Barnes, 1987) taking into account GC base composition and

problem homology with known sequence data of template DNA. Oligonucleotides were synthesised on the Cyclone DNA Synthesizer (BioSearch). The oligonucleotides were removed from the columns using 1 ml 30% ammonia followed by an overnight incubation at 55°C for base deprotection. The ammonia was then evaporated off, and the pellet washed twice in 100% ethanol and resuspended in loading buffer (38% (v/v) formamide, 8.0 mM EDTA, 0.02% Bromophenol Blue, 0.02% Xylene Cyanol FF). The oligonucleotide was then size-purified on a 16% acrylamide, 8.0 M urea denaturing gel (see section 2.4.3) and eluted from the gel matrix by incubating the gel slice in $ddH_2O$ overnight at room temperature. The oligonucleotide was then bound to a Sep-Pak C-18 column (Millipore) prewashed with 5 ml of methanol. The column was washed with 10 ml of $ddH_2O$, and the oligonucleotide eluted with 1 ml 0.05 M triethylamine pH 8.0 in 50% (v/v) methanol. The solvent was evaporated under vacuum, the pellet washed once with $ddH_2O$ and then resuspended in $ddH_2O$.

### 2.3.9 Primer Extension

For the primer extension, a 21 bp oligonucleotide complementary to a region 50 bp downstream from the 5' end of the cloned insert DNA was synthesized and size-purified as described in section 2.3.8. The primer (50 $\mu$g) was end-labeled with 40 units of T4 polynucleotide kinase and 200 $\mu$Ci [$\gamma$-$^{32}$P]ATP following the protocol of Arrand (1985). Unincorporated nucleotides were separated by a G-15 spun column. The final specific activity of the primer was 2.5 x

$10^8$ cpm per μg primer as determined by liquid scintillation counting.

10 ng of the labeled primer prepared above and 20 μg of RNA from *Arabidopsis thaliana* were incubated overnight at 30°C in 20 μl of annealing buffer containing 80% (v/v) formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM PIPES, pH 6.5, and 80 units of RNAsin (Promega). After annealing, the material was precipitated with 2 volumes 100% ethanol, the pellet washed twice in 70% ethanol, and resuspended in 30 μl reverse transcription buffer containing 50 mM Tris-Cl, pH 8.3, 10 mM $MgCl_2$, 150 mM KCl, 1 mM dATP, 1 mM dTTP, 1 mM dCTP, 1 mM dGTP, 10 mM DTT, 20 units RNAsin and 27 units Reverse Transcriptase (Promega). Reverse transcription continued for 1 hour at 42°C after which RNA was degraded by incubating the mix for 5 minutes at 100°C in the presence of 0.3 N NaOH. The base was neutralized with 1 μl glacial acetic acid, the DNA was precipitated with ethanol and the pellet was washed once with 70% ethanol and resuspended in loading buffer (38% formamide, 8.0 mM EDTA, 0.02% Bromophenol Blue, 0.02% Xylene Cyanol FF). The primer extension product was analyzed using a 5% sequencing gel alongside a sequencing ladder obtained using the same primer.

### 2.3.10 *In Vitro* Translation

*In vitro* translation of poly $(A)^+$ RNA and of *in vitro* transcribed RNA was carried out using [$^{35}$S]methionine (1000 Ci/mmol) and a rabbit reticulocyte lysate (Promega) following the manufacturer's instructions.

## 2.4 ELECTROPHORESIS

### 2.4.1 Gel Electrophoresis of Protein

One-dimensional SDS-PAGE was performed as described by Laemmli (1970). Concave exponential gradient (9-14%) acrylamide gels were prepared as described by O'Farrell (1975). Protein was detected by Coomassie Blue staining followed by silver-staining with Bio-Rad Silver Staining Kit (Bio-Rad Laboratories (Canada) Ltd). $^{14}C$ was detected fluorographically by incubating the gels in Amplify (Amersham International) before drying and exposure to preflashed X-ray plates. $^{35}S$ and $^{32}P$ were detected autoradiographically. The X-ray plates were scanned with an LKB Bromma 2202 Ultrascan laser densitometer at 633 nm and the signal was recorded and integrated with an LKB Bromma 2220 recording integrator.

### 2.4.2 Gel Electrophoresis of DNA

DNA was electrophoresed on horizontal gels containing 1% agarose, 40 mM Tris-acetate pH 8.0, 1 mM EDTA and 0.5 μg/ml ethidium bromide as described by Maniatis *et al.* (1982).

### 2.4.3 Gel Electrophoresis of Oligonucleotides

Custom-synthesized oligonucleotides were size-purified on 1.5 mm thick, 16% polyacrylamide, 8.0 M urea gels buffered in 100 mM Tris, 83 mM boric acid and 1 mM EDTA. The gels were prerun at 1000 V until the temperature of the glass plates was

above 45°C. A water cooling system ensured that the temperature would not rise above 60°C during the two hour run.

### 2.4.4 Gel Electrophoresis for Sequencing

Sequencing reactions were separated on 5.0% polyacrylamide, 8.0 M urea, 0.4-0.8 mm wedge gels buffered in 100 mM Tris, 83 mM boric acid and 1 mM EDTA (Davis *et al.* 1986). Gels were prerun at 1500 V until the temperature of the glass plates was above 40°C. After electrophoresis, the urea was removed by incubation in 10% (v/v) acetic acid and 12% (v/v) methanol for 45 minutes after which time the gels were dried at 80°C for 1 hour. Sequencing reactions were visualized by autoradiography.

## 2.5 QUANTIFICATION

### 2.5.1 Protein Assays

To avoid interference from added Triton X-100 and phospholipid, protein was estimated by a modification of the method of Peterson (1977). Since the 0.015% (w/v) deoxycholate employed to solubilize membrane proteins in the standard method of Peterson was relatively ineffective with tonoplast, the samples (50-200 $\mu$l) were made 4% (w/v) with Triton X-100 before bringing the volume to 1 ml with distilled water and adding 100 $\mu$l of 0.15% (w/v) deoxycholate. This modification enabled the direct comparison of the protein content of native membranes with those of solubilized preparations. The method of Peterson

otherwise grossly underestimated the protein content of native membranes.

2.5.2 Betanin Estimations

Residual betanin associated with the tonoplast vesicles and solubilized membranes was measured at $A_{535nm}$.

2.5.3 Quantification of Nucleic Acids

Nucleic acids were quantified at 260 nm in a Perkin-Elmer spectrophotometer assuming an absorbance of 1.0 corresponds to 40 μg/ml RNA, 50 μg/ml double stranded DNA, or 20 μg/ml short (i.e., less than 21 bp) single stranded DNA.

# Chapter 3. IDENTIFICATION OF 3-O-(4-BENZOYL)BENZOYLADENOSINE 5'- TRIPHOSPHATE- AND N,N'-DICYCLOHEXYLCARBODIIMIDE-BINDING SUBUNITS OF A HIGHER PLANT $H^+$-TRANSLOCATING TONOPLAST ATPase

## 3.1 ABSTRACT

The polypeptide composition of the $NO_3^-$-sensitive $H^+$-ATPase of vacuolar membrane (tonoplast) vesicles isolated from red beet (*Beta vulgaris* L.) storage root was investigated by affinity labeling with [$\alpha$-$^{32}$P]3-O-(4-benzoyl)benzoyladenosine 5'-triphosphate ([$\alpha$-$^{32}$P]BzATP) and [$^{14}$C]N,N'-dicyclohexyl-carbodiimide ([$^{14}$C]DCCD). The photoactive affinity analog of ATP, BzATP, is a potent inhibitor of the tonoplast ATPase, (apparent $K_I$ = 11 $\mu$M) and the photolysis of [$\alpha$-$^{32}$P] BzATP in the presence of native tonoplast yields one major $^{32}$P-labeled polypeptide of 57kDa. Photoincorporation into the 57-kDa polypeptide shows saturation with respect to [$\alpha$-$^{32}$P]BzATP concentration and is blocked by ATP. [$^{14}$C]DCCD, a hydrophobic carboxyl reagent and potent irreversible inhibitor of the tonoplast ATPase ($k_{50}$=20 $\mu$M) labels a 16-kDa polypeptide in native tonoplast.

The tonoplast ATPase is purified approximately 12-fold by Triton X-100 solubilization and Sepharose 4B chromatography. Partial purification results in the enrichment of two prominent polypeptides of 67 and 57 kDa. Solubilization, chromatography,

and sodium dodecylsufate-polyacrylamide gel electrophoresis of tonoplast labeled with [$\alpha$-$^{32}$P]BzATP or [$^{14}$C]DCCD results in co-purification of the 57- and 16-kDa labeled polypeptides with ATPase activity.

It is concluded that the tonoplast $H^+$-ATPase is a multimer containing structurally distinct BzATP- and DCCD-binding subunits of 57 and 16 kDa, respectively. The data also suggest the association of a 67-kDa polypeptide with the ATPase.

## 3.2 INTRODUCTION

Two main categories of membrane-bound $H^+$-translocating ATPases have been functionally and structurally characterized in microorganisms, plants, and animal cells. $H^+$-ATPases of the first category are located in the plasma membranes of eucaryotic microorganisms and plants (Serrano, 1984). These $H^+$-ATPases catalyze an essentially irreversible reaction, are subject to inhibition by vanadate, and consist of only one major 100-kDa subunit (Scarborough and Addison, 1984) which forms a phosphorylated acyl intermediate during ATP hydrolysis. The plasma membrane-type $H^+$-ATPases are considered to be analogous to the Na+,$K^+$-ATPase, sarcoplasmic reticulum $Ca^{2+}$-ATPase, and gastric $H^+$,$K^+$-ATPase of animal cells (Carafoli and Scarpa, 1982). The second category consists of the $F_1F_0$-ATPases of mitochondria, chloroplasts, and procaryote plasma membranes. These enzymes catalyze a freely reversible reaction, are subject to inhibition by azide but not vanadate,

consist of several distinct subunits, and do not form a phosphorylated intermediate during ATP hydrolysis (Maloney, 1982). Whereas the 100-kDa polypeptide of the plasma membrane-type ATPases appears to catalyze both ATP hydrolysis and $H^+$ translocation, there is a partitioning of function between the subunits of the $F_1F_0$ complex such that one sector ($F_1$) functions as an ATPase while another, transmembranous, sector ($F_0$) functions as a $H^+$ channel.

Not all $H^+$-ATPase fall into these two categories, however. Recent investigations have shown that vacuolar membrane (tonoplast) (Kakinuma *et al.*, 1981; Bowman and Bowman, 1982; Poole *et al.*, 1984; Uchida *et al.*, 1985), lysosomes (Schneider, 1981; Ohkuma *et al.*, 1982), chromaffin granules and synaptosomes (Carafoli and Scarpa, 1982; Cidon *et al.*, 1983), clathrin-coated vesicles (Xie *et al.*, 1984) and endosomes (Galloway *et al.*, 1983) contain $H^+$-ATPases which are insensitive to both vanadate and azide and therefore appear to be distinct from the plasma membrane-type and $F_1F_0$ categories of $H^+$-ATPase. Whether these enzymes represent a third category of $H^+$-ATPases is not known, but the limited data concerning their anion requirements, association with endomembranes with an acidic interior, and established packaging function is at least consistent with the notion of a third category.

In this paper we describe the solubilization, partial purification, and affinity labeling of the tonoplast $H^+$-ATPase of *Beta vulgaris* L. with [$\alpha$-$^{32}$P]3-O-(4-benzoly)benzoyladenosine 5'-triphosphate(BzATP), a photoaffinity analog of ATP (Williams and Coleman, 1982), and [$^{14}$C]N,N'-dicyclohexylcarbodiimide

(DCCD), a hydrophobic carboxyl reagent known to bind to the DCCD-binding protein of the $F_0$ component of the membrane sector of the $F_1F_0$ complex (reviewed in Linnett and Beechey, 1979). The results demonstrate that this tonoplast $H^+$-ATPase is structurally distinct from the plasma membrane and $F_1F_0$ ATPases and that the BzATP- and DCCD-binding sites were situated on different polypeptides within a multimeric complex.

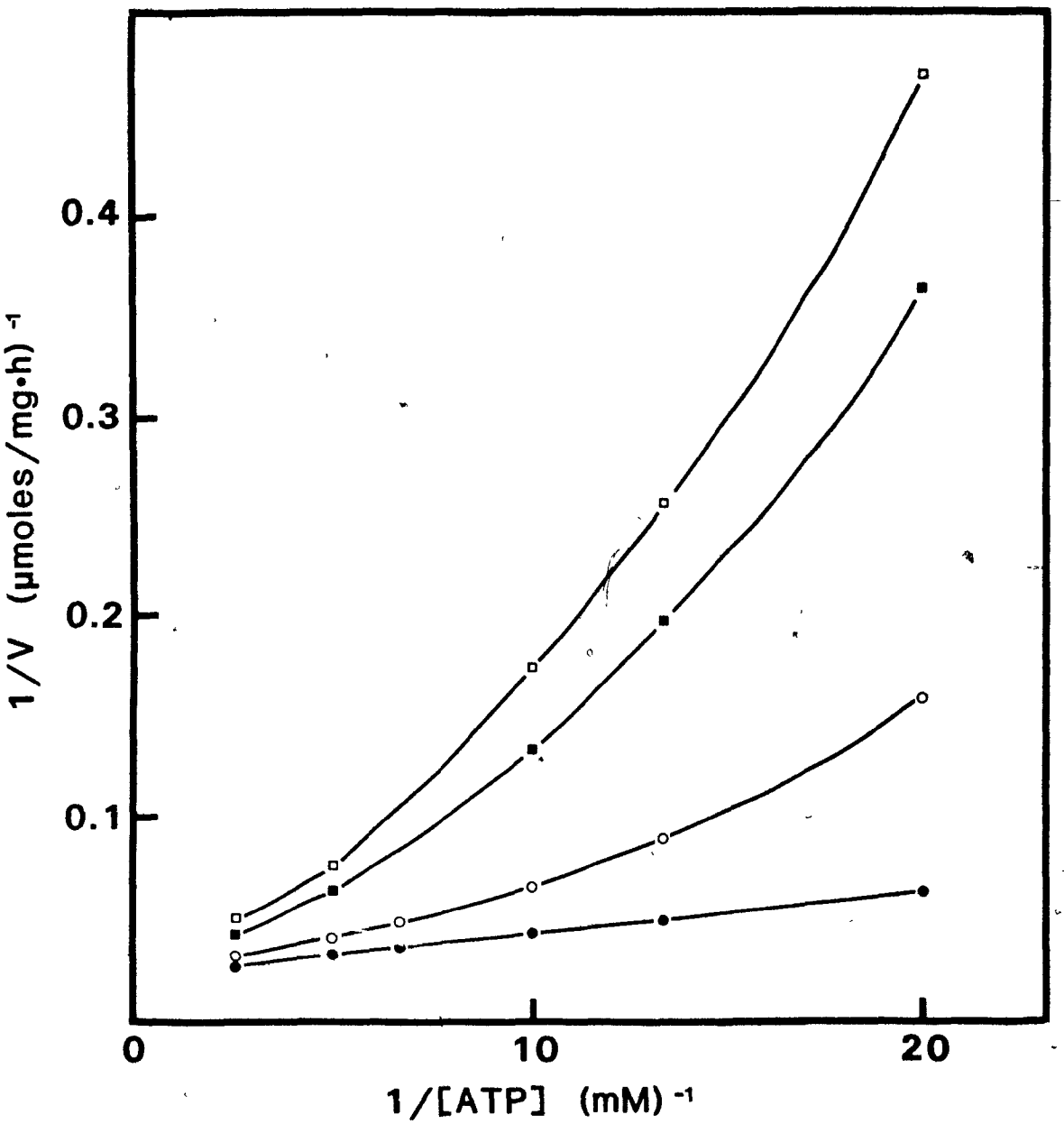
## 3.3 RESULTS

### 3.3.1 Native Tonoplast

#### 3.3.1.1 BzATP as an Inhibitor of Tonoplast ATPase

The efficacy of BzATP as a substrate analog for the $H^+$-ATPase of native tonoplast is indicated by the results in Fig. 3.1. In the absence of irradiation, BzATP behaves somewhat like a competitive inhibitor insofar as a constant $V_{max}$ is approached at high ATP concentrations. Although BzATP caused the tonoplast ATPase to deviate from Michaelis-Menten kinetics with respect to ATP concentration, in that BzATP was disproportionately inhibitory at low ATP concentrations, a secondary plot of the data for higher ATP concentrations, where Michaelis-Menten kinetics are approximated, yielded an apparent $K_I$ of 10 $\mu$M. 4-benzoylbenzoic acid had no effect on ATPase activity, suggesting that the ATP moiety of BzATP is the structural determinant required for inhibition, and BzATP, alone, underwent negligible hydrolysis when incubated with tonoplast (data not shown).

Figure 3.1 **Effect of BzATP on ATPase activity of native tonoplast.** ATPase activity was measured in a coupled system as described under "Materials and Methods" except that $MgSO_4$ was present at a concentration of 0.4 mM. Reciprocal steady-state ADP formation in the absence (•) and presence of 10 $\mu$M (○), 30 $\mu$M (■), and 50 $\mu$M (□) BzATP.


0.4
0.3
0.2
0.1
1/V (µmoles/mg•h) -1
0
10
20
1/[ATP] (mM) -1

3.3.1.2 Covalent Photolabeling of Native Membranes with [α-$^{32}$P]BzATP

The pattern of labeling of native tonoplast with [α-$^{32}$P]BzATP upon irradiation with UV light was found to be strictly dependent on the concentration of [α-$^{32}$P]BzATP employed. Initial experiments in which labeling was performed with 10 μM [α-$^{32}$P]BzATP yielded two labeled polypeptides upon SDS-PAGE and autoradiography: a major $^{32}$P-labeled band with a molecular size of 57 kDa and a minor 37 kDa band. Subsequent analyses of the concentration dependence of the labeling of these two bands, by densitometry of autoradiograms such as that shown in Fig. 3.3, demonstrated that the two polypeptides labeled with different kinetics. The number of counts incorporated into the 57-kDa component saturated at 10 μM [α-$^{32}$P]BzATP, but incorporation into the 37-kDa component showed no indication of saturation (Fig. 3.2A). These data, together with the finding that 50 μM ATP in the reaction medium during photolysis abolished [α-$^{32}$P]BzATP-mediated labeling of the 57- but not the 37-kDa polypeptide (Fig. 3.2B), suggested that labeling of the 37-kDa band resulted from the nonspecific binding. All subsequent labeling experiments were therefore performed with 5 μM [α-$^{32}$P]BzATP to give near-saturating labeling of the 57-kDa component, while minimizing nonspecific labeling of the 37-kDa, and possibly other, components (see Fig. 3.3 for typical autoradiogram in which 5 μM [α-$^{32}$P]BzATP was used for covalent labeling).

Figure 3.2 **Kinetics of photoincorporation of [α-$^{32}$P]BzATP into 57- (O) and 37-kDa (•) polypeptides of native tonoplast.** A, influence of [α-$^{32}$P]BzATP concentration on photoincorporation; B, influence of ATP concentration on photoincorporation of 5 μM [α-$^{32}$P]BzATP. After exposure to gels, the x-ray plates were scanned at 633 nm and the absorbance was integrated with a recording integrator.

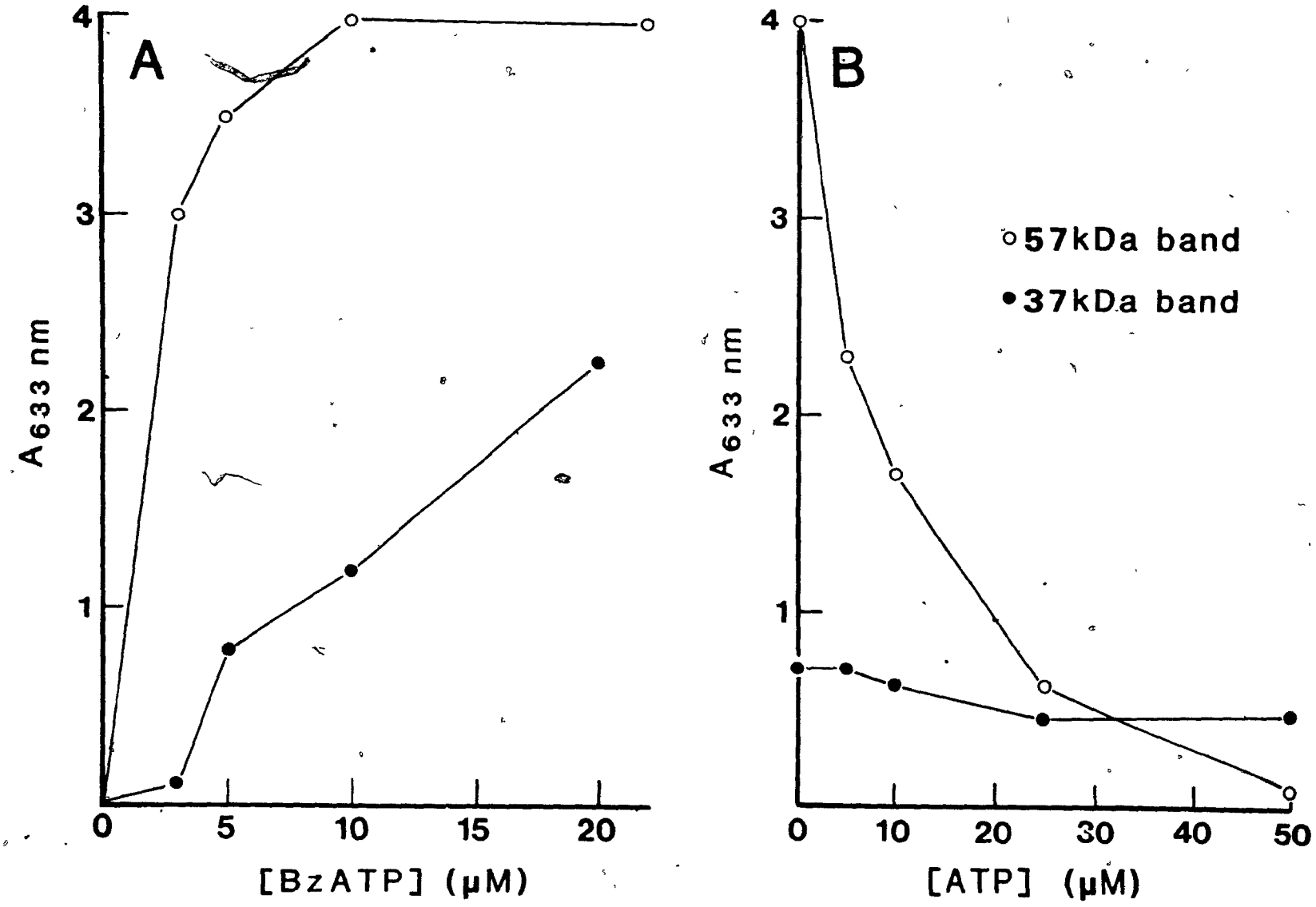
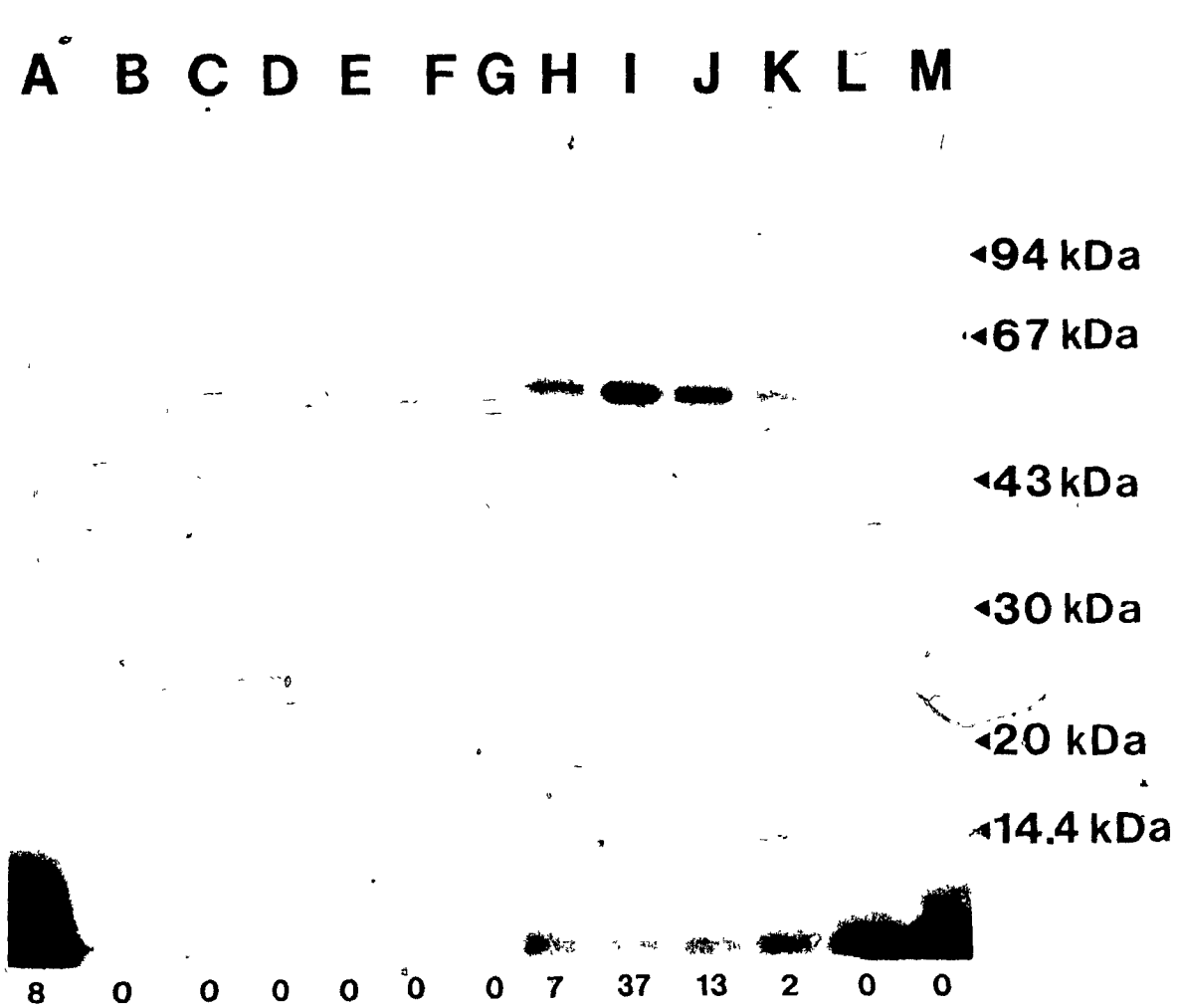

A
B
4
3
2
1
0
5
10
15
20
30
40
50
$A_{633\ nm}$
[BzATP] (μM)
[ATP] (μM)
57kDa band
37kDa band

Figure 3.3 **Identification of the polypeptides labeled by photoirradiation of [α-$^{32}$P]BzATP.** Tonoplast was photoirradiated in the presence of 5 μM [α-$^{32}$P]BzATP, solubilized with 4% (w/v) Triton X-100 and subjected to Sepharose CL-4B chromatography. 15-μg aliquots of protein were subjected to SDS-PAGE on 0.75-mm concave exponential gradient (9-14%) acrylamide slab gels. $^{32}$P label was visualized autoradiographically. Lane A, protein from native tonoplast; lanes B-M, consecutive pairs of fractions from Sepharose CL-4B chromatography starting from fraction number 24 and ending at fraction number 48 (Fig. 3.7). Each pair of fractions was pooled before preparation for SDS-PAGE. Lanes B, I, and L correspond to peak I, peak ATPase, and peak II, respectively, in Fig. 3.7. The numbers under the lanes represent the specific activities of the ATPase in the corresponding chromatographic fractions.


A B C D E F G H I J K L M
94 kDa
67 kDa
43 kDa
30 kDa
20 kDa
14.4 kDa
8 0 0 0 0 0 0 7 37 13 2 0 0
ATPase activity (μmol/mg·h)

### 3.3.1.3 Covalent Labeling of Native Membranes with [$^{14}$C]DCCD

It is well established that DCCD is a potent inhibitor of $H^+$-ATPases (Racker, 1976) and the tonoplast ATPase is no exception. The ATPase of native membranes was 50% inhibited by 20 $\mu$M DCCD (Table 3.1) and the incubation of tonoplast in 30 $\mu$M [$^{14}$C]DCCD yielded one $^{14}$C-labeled polypeptide with an apparent molecular size of 16 kDa (Fig. 3.4, lane A).

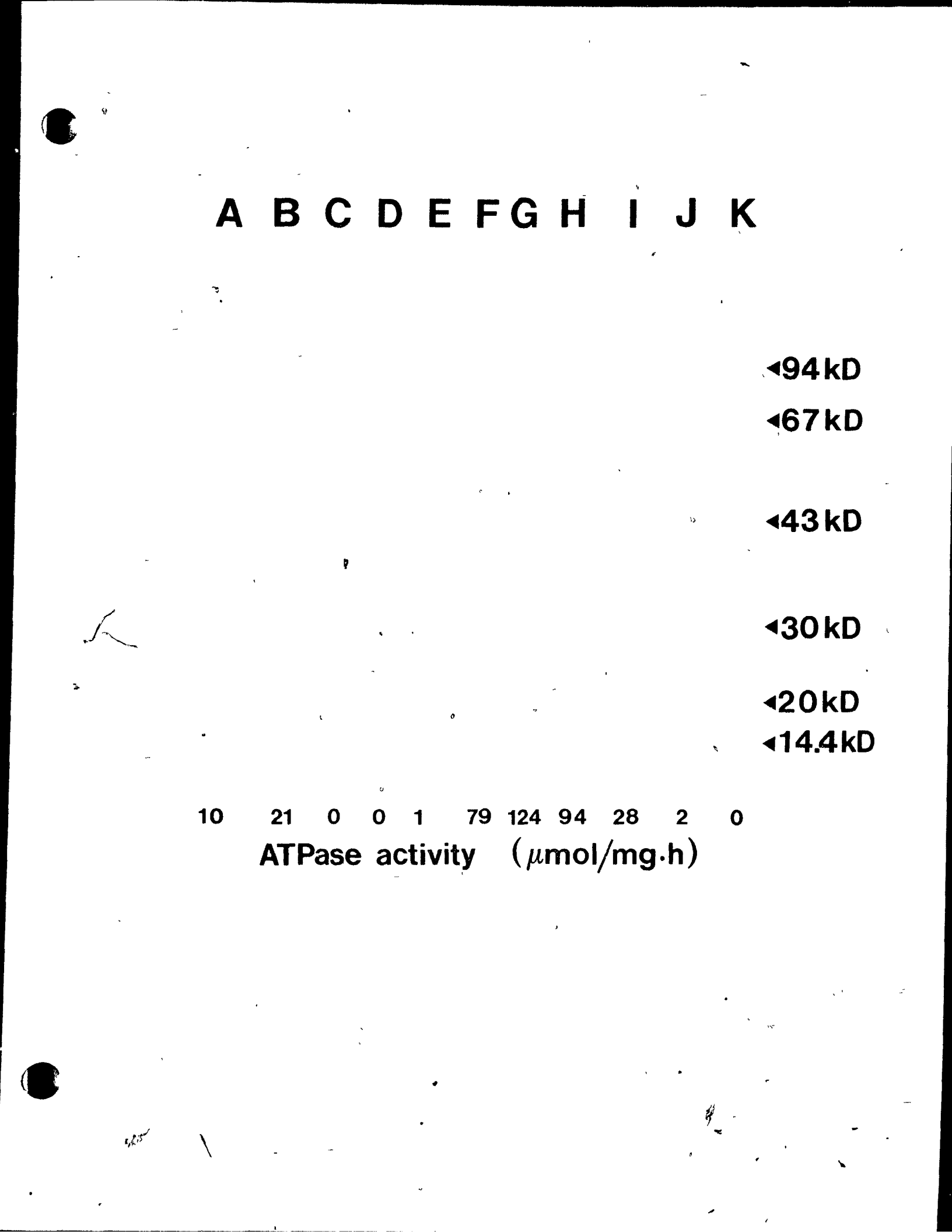
## 3.3.2 Partially Purified ATPase

In view of the complex polypeptide composition of the protein from native tonoplast vesicles, independent criteria for the association of a given binding site with the ATPase were required. Unlabeled, [$\alpha$-$^{32}$P]BzATP-labeled and [$^{14}$C]DCCD-labeled tonoplast vesicles were therefore solubilized, and the ATPase was partially purified with Sepharose 4B chromatography to determine if the $^{32}$P-labeled and $^{14}$C-labeled polypeptides co-purify with phosphohydrolase activity.

**Table 3.1 Kinetics and inhibitor sensitivities of native tonoplast and partially purified ATPase.** The kinetics of ATP hydrolysis were determined in a coupled system containing 0-3 mM ATP, 50 mM KCl, 3 mM $MgSO_4$ and 30 mM Tris-Mes buffer (pH 8.0). For the inhibitor studies, native tonoplast and the peak ATPase fractions from Sepharose 4B chromatography were incubated on ice for 1 h with the indicated concentrations of the inhibitors. The assay reaction was initiated by the addition of 3 mM Tris-ATP and allowed to proceed for 30 min at 37°C.

| | Native tonoplast | Partially purified ATPase |
|---|---|---|
| $K_m$ (mM) | 0.09 | 0.10 |
| $V_{max}$ ($\mu$mol/mg h) | 25.6 | 322.6 |
| $k_{50}$ ($NO_3^-$) (mM) | 31.6 | 15.8 |
| $k_{50}$ (DCCD) ($\mu$M) | 20.0 | 63.1 |
| | Inhibition | |
| | % | |
| Control | 0 | 0 |
| 100 mM $KNO_3$ | 70 | 98 |
| 100 $\mu$M vanadate | 13 | 4 |
| 100 $\mu$M molybdate | 1 | 2 |
| 1 mM azide | 0 | 0 |

Figure 3.4. **Identification of the polypeptides labeled with** [$^{14}$C]DCCD. Tonoplast was incubated in 30 $\mu$M [$^{14}$C]DCCD for 25 min at room temperature, solubilized with 4% (w/v) Triton X-100, and subjected to Sepharose 4B chromatography. Ten $\mu$g aliquots of protein were electrophoresed as described in the legend to Fig. 3.3 and $^{14}$C label was visualized fluorographically. Lane A, protein from native tonoplast; lane B, Triton X-100-solubilized tonoplast protein; lanes C-K, consecutive pairs of fractions from Sepharose chromatography. Each pair of fractions was pooled before preparation for SDS-PAGE. Lanes C, G, and K correspond to peak I, peak ATPase activity, and peak II, respectively, in Fig. 3.7, but note that the fraction numbers do not exactly correspond since this material was chromatographed on a Sepharose 4B, not a Sepharose CL-4B, column. The numbers under the lanes represent the specific activities of ATPase in the corresponding chromatographic fractions.


A B C D E F G H I J K
94kD
67kD
43kD
30kD
20kD
14.4kD
10 21 0 0 1 79 124 94 28 2 0
ATPase activity (μmol/mg·h)

### 3.3.2.1 Solubilization of ATPase

Tonoplast protein was solubilized with Triton X-100 (Fig. 3.5). Only at Triton X-100 concentrations of less than 0.5% was the ATPase solubilized in active form and not dependent on added phospholipid for maximal activity (Figs. 3.5 and 3.6). The amount of activity recovered and the degree of phospholipid activation was maximal at 1.0% Triton X-100 (Figs. 3.5 and 3.6). The addition of 1.5 mg/ml phospholipid to the assay medium increased the activity by 3-fold and, when assayed in the presence of added phospholipid, the 1.0% Triton X-100-solubilized ATPase was approximately 2-fold more active than native tonoplast. Although Triton X-100 concentrations of 1.25-4.0% did not yield as active a preparation as 1.0% Triton X-100, 80% of the maximal solubilized activity was conserved throughout the higher concentration range and 1.5 mg/ml phospholipid caused a 3-fold stimulation. Thus to ensure thorough solubilization and delipidation of the tonoplast ATPase, a Triton X-100 concentration of 4.0% was routinely employed.

### 3.3.2.2 Sepharose 4B Chromatography

Chromatography of the solubilized membranes on a Sepharose CL-4B column equilibrated and eluted with running buffer containing 0.3% Triton X-100 and 0.05 mg/ml phospholipid yielded two major protein peaks (peaks I and II) and a peak of ATPase activity between the two (Fig. 3.7). Preliminary experiments demonstrated that it was imperative that

Figure 3.5 **Solubilization of tonoplast with Triton X-100.** The samples (2 mg/ml of membranae protein) were incubated on ice for 20 min, centrifuged at 200,000 x g for 35 min, and protein (■) and ATPase with (•) and without (O) the addition of 1.5 mg/ml sonicated phospholipid to the assay medium were measured in the supernatant. Thirty μl aliquots of the supernatant were assayed for ATPase activity in a 1 ml reaction volume by the coupled method. The specific activity of the native membranes was 26.3 μmol/mg h.

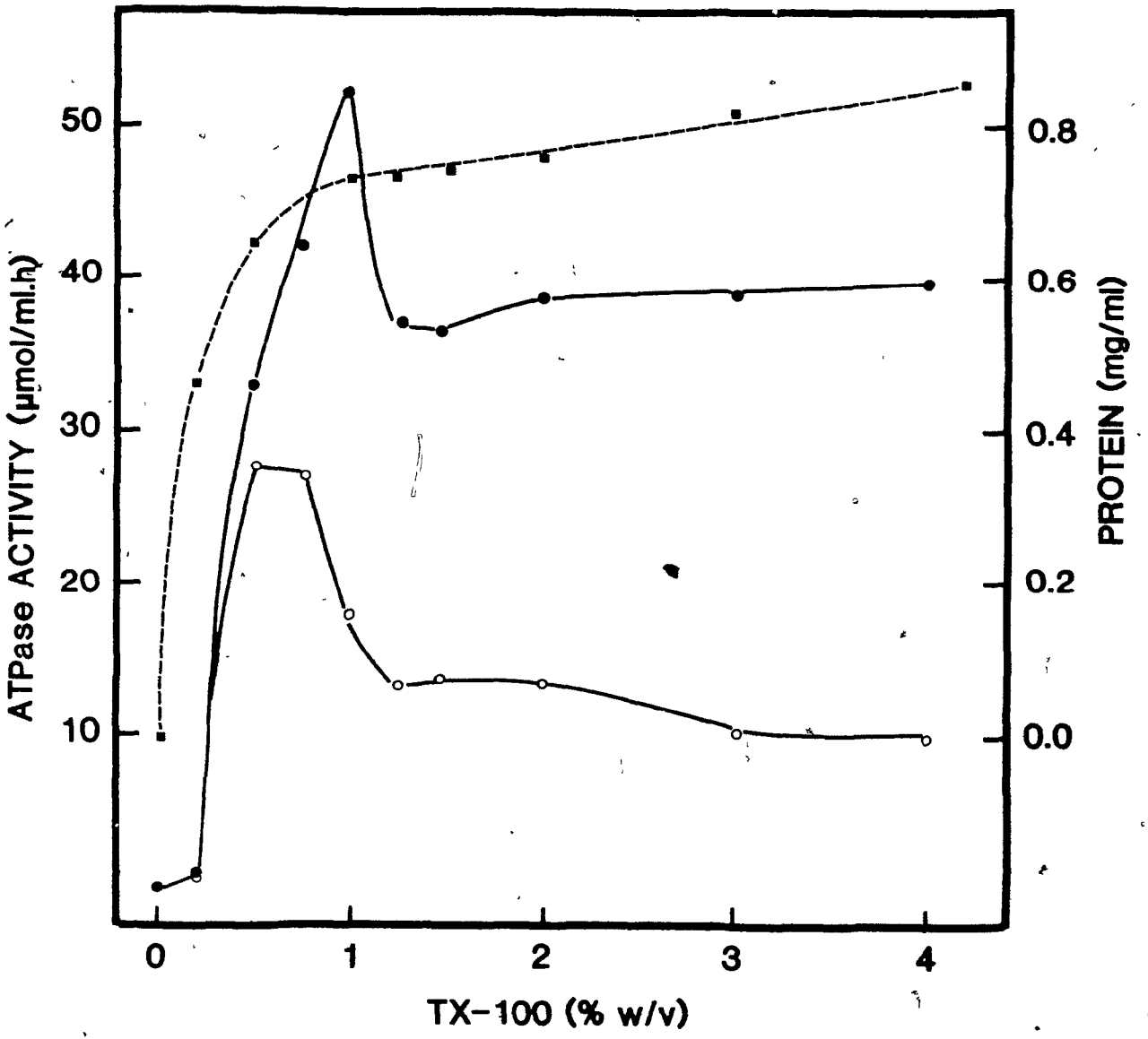

ATPase ACTIVITY (µmol/ml.h)
PROTEIN (mg/ml)
TX-100 (% w/v)
50
40
30
20
10
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4

Figure 3.6 **Phospholipid activation of solubilized tonoplast ATPase.** The ATPase was solubilized and activity was measured in the presence of 0.0-2.0 mg/ml sonicated phospholipid as described in the legend to Fig. 3.5. TX-100 = Triton X-100.

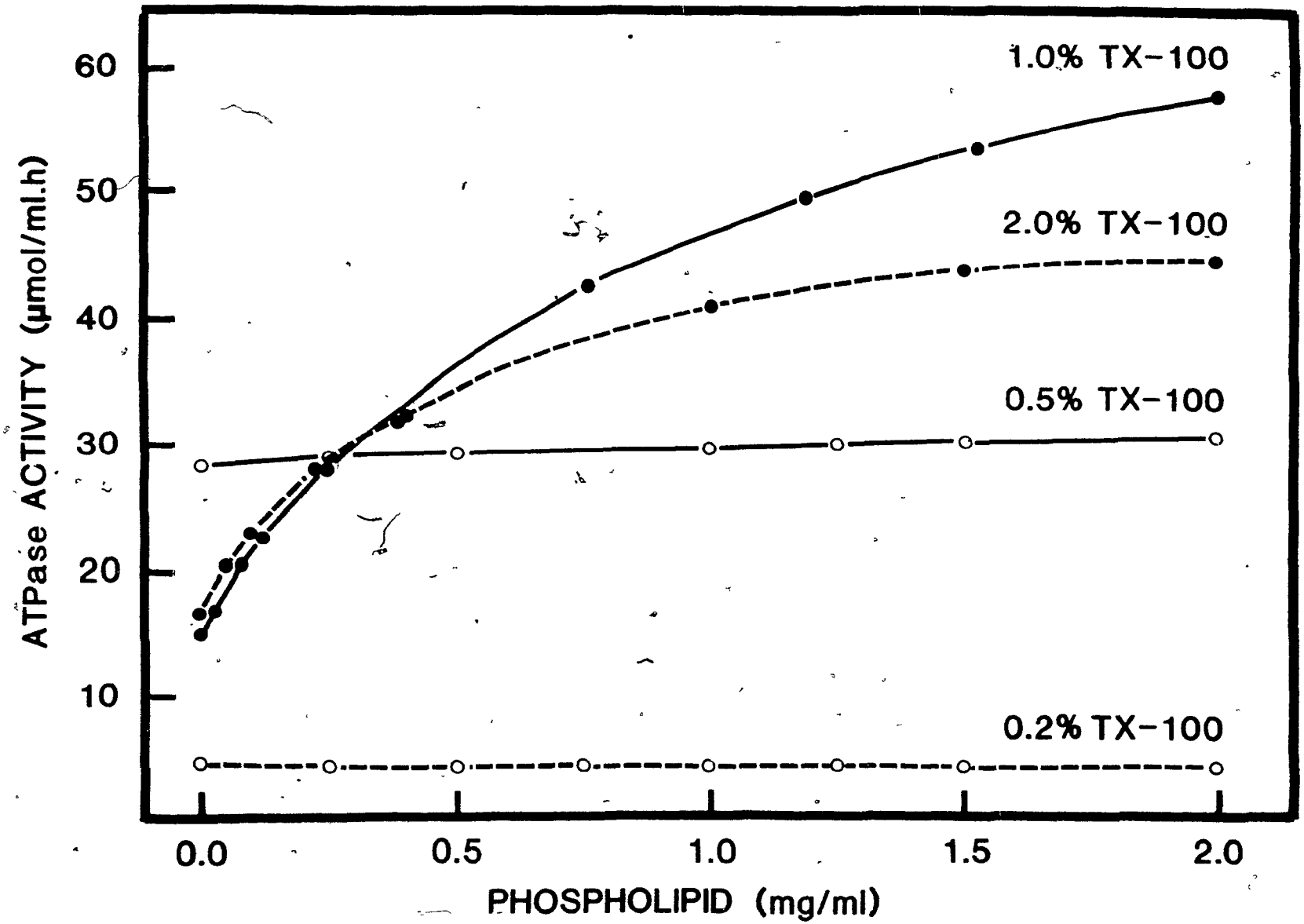
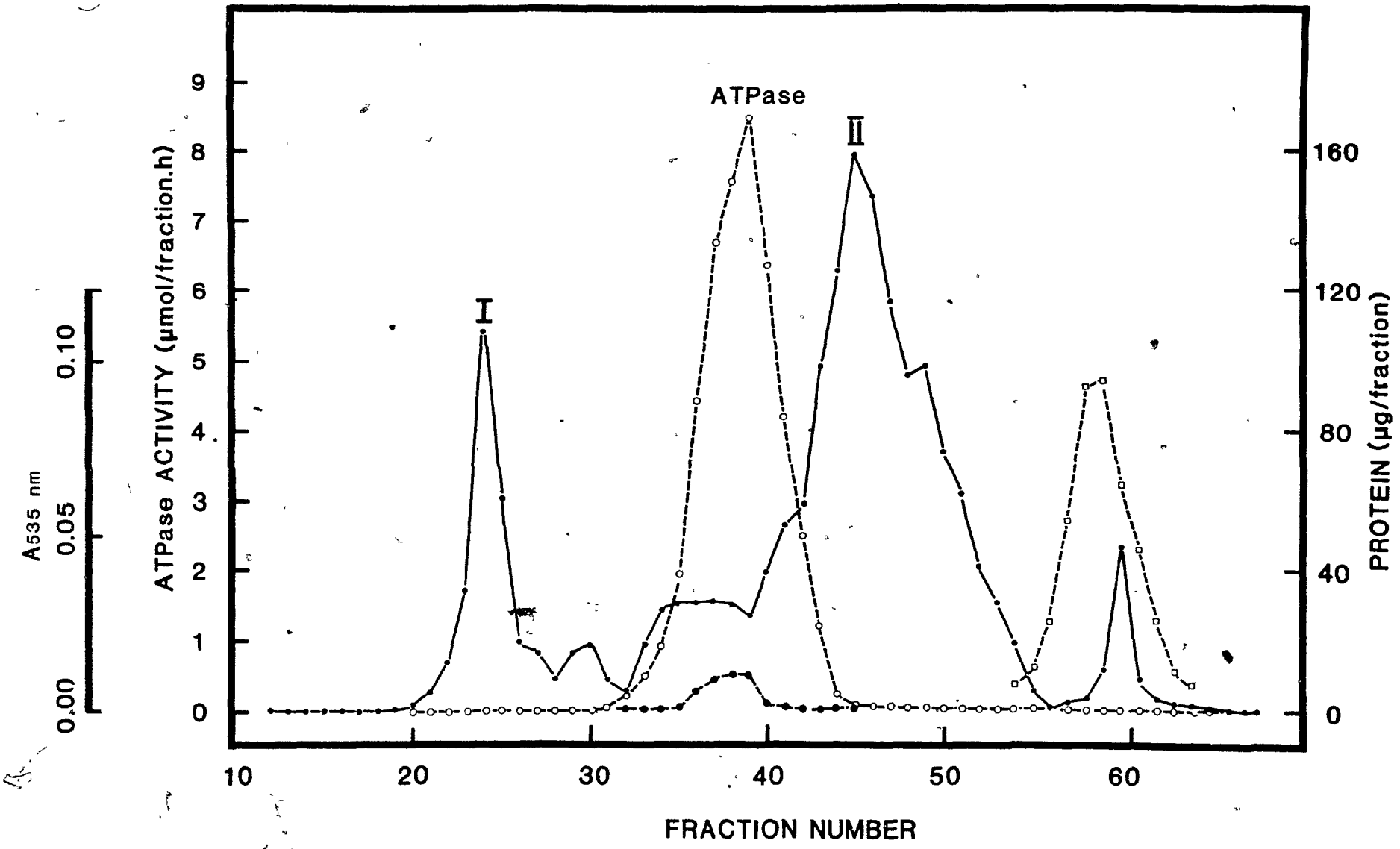

1.0% TX-100
2.0% TX-100
0.5% TX-100
0.2% TX-100
ATPase ACTIVITY (µmol/ml.h)
60
50
40
30
20
10
0.0
0.5
1.0
1.5
2.0
PHOSPHOLIPID (mg/ml)

Figure 3.7 **Chromatogram of solubilized tonoplast on Sepharose CL-4B column.** Protein (•——•), betanin ($A_{535}$ $n_m$; □- - -□), and ATPase activity in the presence (O- - -O) or absence (•- - -•) of 2 mg/ml sonicated phospholipid are shown. ATPase activity was measured with 30 $\mu$l aliquots of the fractions in a reaction volume of 300 $\mu$l by the Ames method (1966).


ATPase
I
II
ATPase ACTIVITY (µmol/fraction.h)
PROTEIN (µg/fraction)
A535 nm
0.00
0.05
0.10
0
1
2
3
4
5
6
7
8
9
0
40
80
120
160
10
20
30
40
50
60
FRACTION NUMBER

phospholipid be added to the running buffer in order to obtain quantitative recovery of the applied activity. The chromatographic behaviour of the solubilized membranes was similar whether 0.05, 0.50 or 1.00 mg/ml phospholipid was included in the running buffer, but the addition of phospholipid to the assay media was required for maximal ATPase activity when a phospholipid concentration of 0.05 mg/ml was employed (Fig. 3.7). The addition of phospholipid after chromatography in the complete absence of phospholipid did not restore activity, indicating irreversible denaturation of the enzyme.

Table 3.2 summarizes the recoveries and activities of the tonoplast ATPase obtained by solubilization with Triton X-100 and Sepharose 4B chromatography. Generally, the specific activity of the solubilized ATPase was twice that of the native membranes when the former was measured in the presence of added phospholipid. Most of the increase in activity seen upon solubilization resulted from detergent activation rather than differential protein solubilization, as approximately 90% of the original membrane protein was solubilized by 4% Triton X-100. Part of the observed detergent activation probably corresponds to an increase in the accessibility of enzyme to substrate, but the possibility of a detergent-mediated modification of intrinsic rate constants (Briskin and Poole, 1984) cannot be excluded.

The peak ATPase fractions from Sepharose 4B chromatography had an average specific activity of 360 μmol/mg.h, which represents a 12-fold increase over the specific activity of the

Table 3.2 **Partial purification of tonoplast ATPase.** Native tonoplast was solubilized with a final concentration of 4% (w/v) Triton X-100 and subjected to Sepharose 4B chromatography as described under "Materials and Methods." The native membranes were assayed for ATPase in the presence of 5 $\mu$M gramicidin D to ensure $H^+$/cation equilibration. The solubilized membranes were assayed in the presence of 2 mg/ml sonicated phospholipid. The total protein recovered from the Sepharose 4B column (2.14 mg) was essentially the same as the amount applied (2.05 mg). "Total ATPase" refers to the total activity and protein recovered from all the column fractions which had detectable ATPase activity (fractions 31-45 in Fig. 3.7) "Peak ATPase" refers to the ATPase activity of the column fraction containing the greatest specific activity (fraction 38 in Fig. 3.7).

| | Protein | | ATPase activity | | Specific Activity |
|---|---|---|---|---|---|
| | mg | % | $\mu$mol/h | % | $\mu$mol/mg.h |
| Tonoplast | 2.26 | 100.0 | 68.0 | 100.0 | 30.1 |
| Triton X-100 supernatant | 2.05 | 90.7 | 122.9 | 180.7 | 60.0 |
| Sepharose 4B | | | | | |
| Total ATPase | 0.55 | 24.3 | 63.1 | 92.7 | 114.7 |
| Peak ATPase | 0.03 | 1.3 | 10.9 | 16.0 | 363.3 |

native membranes and a 6-fold increase over that of the solubilized preparation. Essentially all of the protein applied to the Sepharose column was recovered, whereas only 51% of the applied solubilized ATPase activity was recovered. Inactivation during chromatography may therefore give a 2-fold underestimate of purification factors.

The identity of the partially purified ATPase with the ATPase in native tonoplast vesicles was confirmed by their inhibitor sensitivities and $K_m$ values (Table 3.1). The activity of the partially purified ATPase was 98% inhibited by 100 mM $KNO_3$ with a $k_{50}$ of 16 mM, whereas the ATPase activity of the original tonoplast vesicles was 70% inhibited at 100 mM $KNO_3$ with a $k_{50}$ of 32 mM. DCCD inhibited both the native membranes and the partially purified enzyme with $k_{50}$ values of 20.0 and 63.1 $\mu$M, respectively. The presence of Triton X-100 and added phospholipid may be responsible for the lower sensitivity of the partially purified enzyme to DCCD (Linnett *et al.*, 1975; Ryrie, 1975). The lack of inhibition of the partially purified enzyme by azide, molybdate and vanadate demonstrated the absence of mitochondrial ATPase (Bowman *et al.*, 1978), nonspecific phosphatase (Leigh and Branton, 1976), and plasma membrane ATPase (O'Neill and Spanswick, 1984), respectively.

The ATPase activities of both the native tonoplast and partially purified enzymes approximated Michaelis-Menten kinetics with respect to ATP concentration and yielded apparent $K_m$ values of 0.09 and 0.10 mM, respectively, in experiments where the ATP concentration was varied but the $Mg^{2+}$

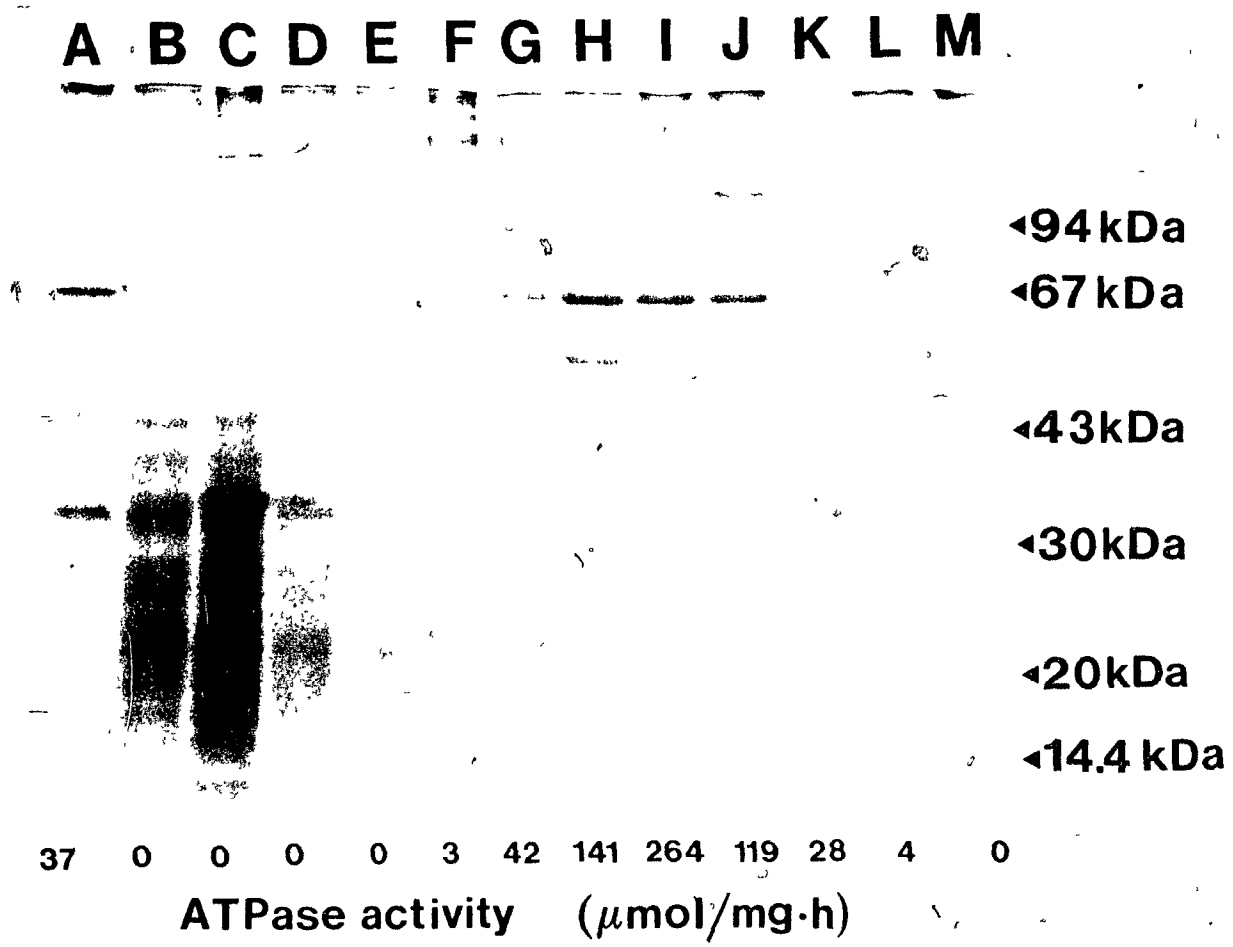
concentration was held constant (Table 3.1).

3.3.2.3 SDS-PAGE

SDS-PAGE of the fractions from Sepharose CL-4B chromatography revealed a substantial enrichment of a 57- and 67-kDa polypeptide in the peak ATPase activity fractions (Fig. 3.8, lanes H, I, and J). Analysis of tonoplast labeled with 5 $\mu$M [$\alpha$-$^{32}$P]BzATP and subjected to Sepharose CL-4B chromatography demonstrated that the 57-kDa polypeptide which co-purified with ATPase activity was the 57-kDa polypeptide which photoincorporated [$\alpha$-$^{32}$P]BzATP (Fig. 3.3). Lanes H, I, and J, which showed maximal $^{32}$P incorporation, correspond to the fractions in which ATPase activity was maximal. Some $^{32}$P label was also found in polypeptides of 150 and 86 kDa, both of which appeared to co-purify with ATPase activity. The intense labeling found at the front of the gel in lane A, and increasing in amount from lane H onwards, represents endogenous phospholipid labeled with $^{32}$P; it does not appear if the protein is extracted with ether before electrophoresis.

Chromatography, SDS-PAGE, and fluorography of [$^{14}$C]DCCD-labeled tonoplast clearly showed co-purification of the 16-kDa $^{14}$C-labeled polypeptide and ATPase activity (Fig. 3.4, lane G) but some labeling was discernible in the fractions after the activity peak (Fig. 3.4, lanes I and J).

Figure 3.8 **SDS-PAGE analysis of fractions from Sepharose CL-4B chromatography.** Each lane was loaded with 6 μg of protein and the separations were made as described in the legend to Fig. 3.3. Protein was visualized by Coomassie Blue staining followed by silver-staining. Lane A, protein from native tonoplast; lanes B-M, consecutive pairs of fractions from Sepharose 4B chromatography, starting from fraction number 24 (Fig. 3.7). Each pair of fractions was pooled before preparation for SDS-PAGE. Lanes B, I, and L correspond to peak I, peak ATPase and peak II, respectively, in Fig. 3.7. The numbers under the lanes represent the specific activities of ATPase in the corresponding chromatographic fractions.


A B C D E F G H I J K L M
94kDa
67kDa
43kDa
30kDa
20kDa
14.4 kDa
37 0 0 0 0 3 42 141 264 119 28 4 0
ATPase activity (μmol/mg·h)

## 3.4 DISCUSSION

We have shown that [$\alpha$-$^{32}$P]BzATP, a photoaffinity analog of ATP, primarily labels a 57-kDa polypeptide in *Beta* tonoplast (Fig. 3.3). BzATP was shown to be a potent inhibitor of ATPase activity with an apparent $K_I$ of 10 $\mu$M (Fig. 3.1). A concentration of 10 $\mu$M [$\alpha$-$^{32}$P]BzATP was sufficient to give maximal labeling of the 57-kDa polypeptide during a 20-min period of UV irradiation, and 50 $\mu$M ATP was sufficient to completely block labeling (Fig. 3.2). Since the 57-kDa polypeptide was also the principal $^{32}$P-labeled component to co-purify with ATPase activity during Sepharose 4B chromatography, it is concluded to be a subunit of the tonoplast ATPase.

The role of the 57-kDa subunit in the ATPase remains to be determined. The observations that (a) ATP protects this component from labeling by [$\alpha$-$^{32}$P]BzATP and (b) BzATP is a potent inhibitor of enzymic activity suggest that the 57 kDa component carries a high affinity binding site for ATP which is essential for catalysis. However, the fact that BzATP is not a simple competitive inhibitor of the ATPase but causes the enzyme to display positive cooperative kinetics with respect to ATP concentration (Fig. 3.1) suggests that it might interact with an ATP binding site distinct from the catalytic site, possibly a regulatory subunit.

A molecular size of 57 kDa for the BzATP binding subunit of the tonoplast ATPase falls within the size range of the $\alpha$ and $\beta$ subunits of various $F_1$-ATPases: 55-62 kDa for the $\alpha$

subunit and 50-56 kDA for the $\beta$ subunit (Vignais and Satre, 1984). Contamination of the tonoplast ATPase by mitochondrial $F_1$-ATPase is, however, ruled out by the complete lack of inhibition of either the native tonoplast or partially purified ATPase by azide (Table 3.1).

The 37-kDa polypeptide which was labeled by [$\alpha$-$^{32}$P] BzATP in native tonoplast showed neither saturation of labeling at low concentrations of [$\alpha$-$^{32}$P]BzATP nor protection by ATP (Fig. 3.2) indicating nonspecific binding. Since this component did not co-purify with ATPase activity during chromatography it appears to be structurally unrelated to the tonoplast ATPase. The minor $^{32}$P-labeled polypeptides of 150 and 86 kDa, on the other hand, did co-purify with ATPase activity. Their significance awaits more homogeneous ATPase preparations as they bind only a small proportion of the total label and are often undetectable autoradiographically.

Incubation of tonoplast with [$^{14}$C]DCCD, at a concentration similar to that required to inhibit the ATPase (Table 3.1), labeled one polypeptide with a molecular size of 16 kDa (Fig. 3.4). This component was solubilized by Triton X-100 and co-purified with ATPase activity during Sepharose 4B chromatography. Since the partially purified ATPase retains sensitivity to DCCD (Table 3.1) and since the 16-kDa polypeptide is the only major $^{14}$C-labeled band showing co-purification with the ATPase, it is concluded that this polypeptide is a subunit of the tonoplast ATPase, and that it carries the DCCD-binding site responsible for ATPase inhibition. The reason for the imperfect correspondence

between ATPase activity and the $^{14}C$-label during chromatography (Fig. 3.4) is not known. Covalent modification of the ATPase with DCCD may alter its chromatographic behaviour, or there may be a partial separation of the 16-kDa subunit from the ATPase upon solubilization and chromatography.

A [$^{14}C$]DCCD-labeled polypeptide migrating between 14 and 21 kDa on SDS gels has also been demonstrated by Bowman (1983) and Uchida *et al.* (1985) in vacuolar membranes from *Neurospora* and *Saccharomyces*, respectively. These polypeptides, like the one identified in the present work, are clearly distinguishable from the DCCD-binding ("c") subunit of the $F_1F_0$ ATPases of *Neurospora* and *Saccharomyces* mitochondria (Sebald *et al.*, 1979; Uchida *et al.*, 1985) and *Escherichia coli* plasma membrane (Schneider and Altendorf, 1984), all of which have a molecular size of 8 kDa. The existence in the tonoplast ATPase of a small DCCD-binding subunit distinct from the 57-kDa ATP-binding subunit is nevertheless reminiscent of the structural dichotomy of the $F_1F_0$ $H^+$-ATPases.

In addition to the 57- and 16-kDa subunits of the tonoplast ATPase identified by labeling with [$\alpha$-$^{32}P$]BzATP and [$^{14}C$] DCCD, respectively, SDS-PAGE of the fractions from Sepharose 4B chromatography (Fig. 3.8) reveals a prominent 67-kDa component which co-purifies with the ATPase. The polypeptides at 57 and 67 kDa in Fig. 3.8 may correspond to the prominent 60- and 70-kDa polypeptides observed by Mandala and Taiz (1985a) in their partially purified tonoplast ATPase preparations from corn coleoptiles. However, association of the 67-kDa polypeptide with the ATPase can only be suggestive

because of the inhomogeneity of the partially purified enzymes studied by Mandala and Taiz (1985a) and ourselves. It is, however, noteworthy that Uchida *et al.*, (1985) have recently found the same basic pattern of subunits for the $H^+$-ATPase of *Saccharomyces cerevisiae* tonoplast; two major 89-and 64-kDa subunits together with a smaller DCCD-binding polypeptide. The tonoplast $H^+$-ATPases of both higher (e.g. *Zea* and *Beta*) and lower plants (e.g. *Neurosopora* and *Saccharomyces*), therefore, appear to have strikingly similar subunit compositions suggesting common membership of a third category of $H^+$-ATPases.

# Chapter 4. EVOLUTION OF ENDOMEMBRANE $H^+$-ATPases: IMMUNOLOGICAL EVIDENCE FOR A COMMON ANCESTOR

## 4.1 ABSTRACT

The evolution of the endomembrane systems of eukaryotic cells can be examined by exploring the evolutionary origins of the endomembrane $H^+$-ATPases. Recent studies suggest that certain polypeptides are common to all $H^+$ pumps of this type. Tonoplast $H^+$-ATPase from *Beta vulgaris* L. was purified and antibodies raised to two of its subunits. Each of these antisera reacted with a polypeptide of the corresponding size in bovine chromaffin granules, bovine clathrin-coated vesicles, and yeast vacuolar membranes, suggesting common structural features and a common ancestor for endomembrane $H^+$-ATPases of different organelles and different phyla. The antiserum raised against the 57 kDa polypeptide of plant tonoplast $H^+$-ATPase also reacted with subunit "a" of the $H^+$-ATPase from the obligately anaerobic bacterium *Clostridium pasteurianum* and to the $\alpha$ subunit of the $H^+$-ATPase from *Escherichia coli*. There was no reactivity with chloroplast or mitochondrial ATPases. These results suggest that endomembrane $H^+$-ATPases may be, in evolutionary terms, primitive $F_0F_1$ ATPases.

## 4.2 INTRODUCTION

The contents of many endomembrane-limited intracellular compartments are maintained at an acidic pH by membrane-bound $H^+$-translocating ATPases. These proton pumps therefore play an essential role in many cellular activities, including the uptake of molecules by receptor-mediated endocytosis, internalization of enveloped viruses, post-translational modification and targeting of proteins, and solute compartmentation (Mellman *et al.*, 1986). Endomembrane $H^+$-ATPases are characterized by their inhibitor sensitivity: they are unaffected by the standard inhibitors of $E_1E_2$-type ATPases (vanadate and ouabain) and of $F_OF_1$-type ATPases (azide, oligomycin and efrapeptin), but show particular sensitivity to sulfhydryl reagents (see section 1.4.2). Endomembrane $H^+$-ATPases resemble $F_OF_1$-type ATPases in being multimeric and electrogenic (probably transporting only $H^+$) and in forming no phosphorylated intermediate. Recent work shows that these ATPases are composed of an integral membrane component forming a proton channel sensitive to DCCD (Sun *et al.*, 1987), and a peripheral nucleotide-binding complex presumed to form the reactive site (Rea *et al.*, 1987a; Steven K. Randall, pers. comm.). A possible evolutionary link between endomembrane and $F_OF_1$-types of ATPases has led to speculation about the evolutionary origin of the endomembrane system as a whole (Maloney and Wilson, 1985; Al-Awqati, 1986). Here we show conservation of structure amongst endomembrane $H^+$-ATPases of plants, fungi and animals, and indicate their possible relationship to bacterial $F_OF_1$ ATPases.

## 4.3 RESULTS AND DISCUSSION

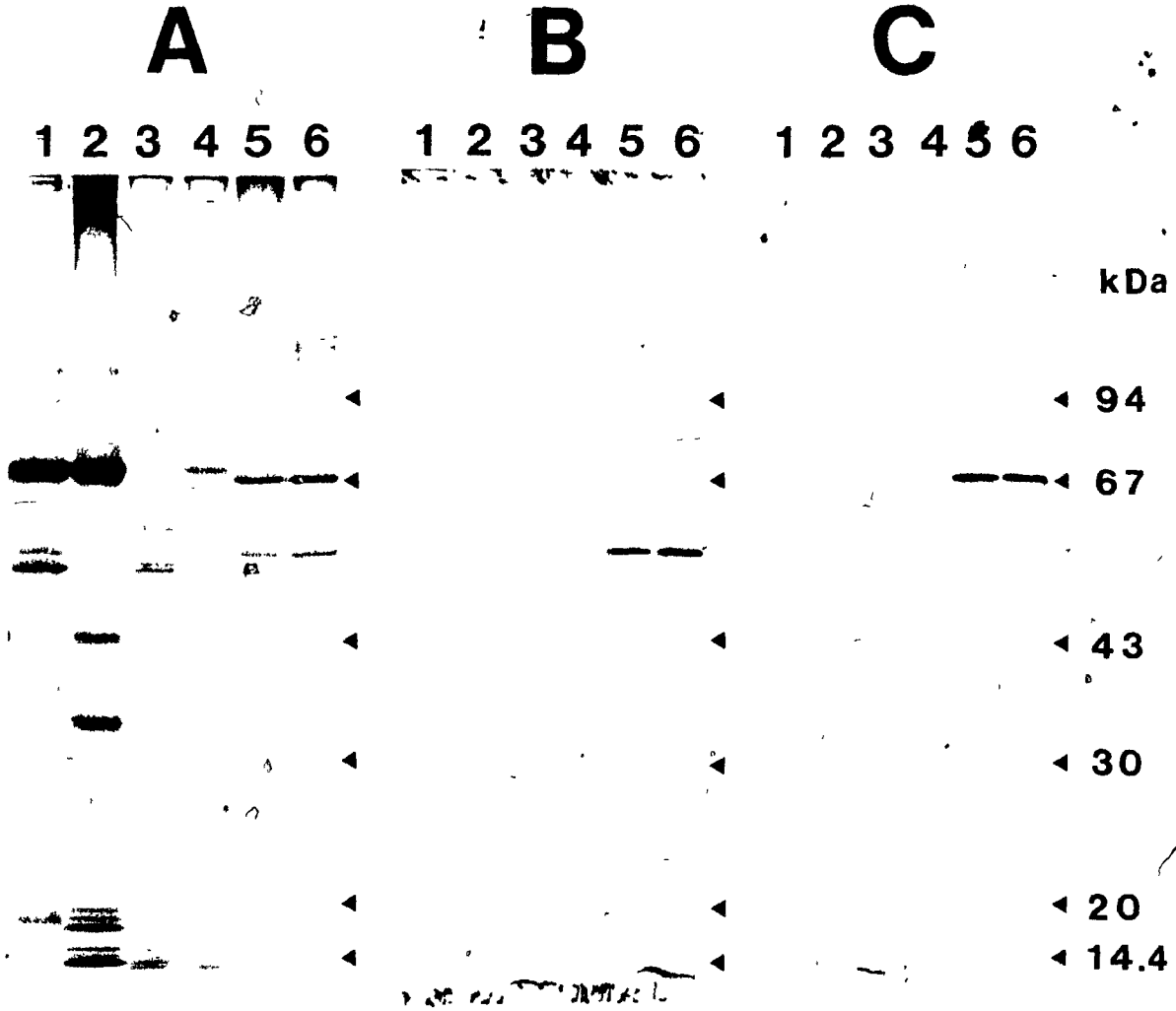
Current information on the structure of endomembrane $H^+$-ATPases from plant, animal, and fungal sources suggests that three polypeptide subunits may be common to all $H^+$-pumps of this type. These are summarized in Table 4.1. To obtain further evidence for subunit homologies, antibodies to the 57 kDa and 67 kDa subunits of beet vacuolar membrane (tonoplast) $H^+$-ATPase were raised as described in section 2.2.1. Evidence for specificity of the resulting antibodies is presented in Figure 4.1. No cross-reactivity was seen with other polypeptides of beet tonoplast, nor with soluble proteins, mitochondria, plasma membrane, or microsomal membranes of beet. The sera were unable to inhibit $H^+$-pumping or hydrolytic activity, nor could they immunoprecipitate the native detergent-solubilized enzyme (data not shown). This was not unexpected as these antisera were raised to SDS-denatured protein.

Table 4.1

**Properties of Subunits Common to Plants, Fungi, and Animals**

| Estimated Molecular Mass (kDa) | Properties |
|---|---|
| 66-80 | Binds nucleotides and SH reagents in several systems. May contain catalytic site. |
| 54-64 | In one study (see Chapter 3) nucleotide binding to this subunit appears essential for ATPase activity. May participate in ATPase reaction. |
| 13-19.5 | Binds the hydrophobic carboxyl reagent DCCD. May form part of a proton channel through the membrane. |

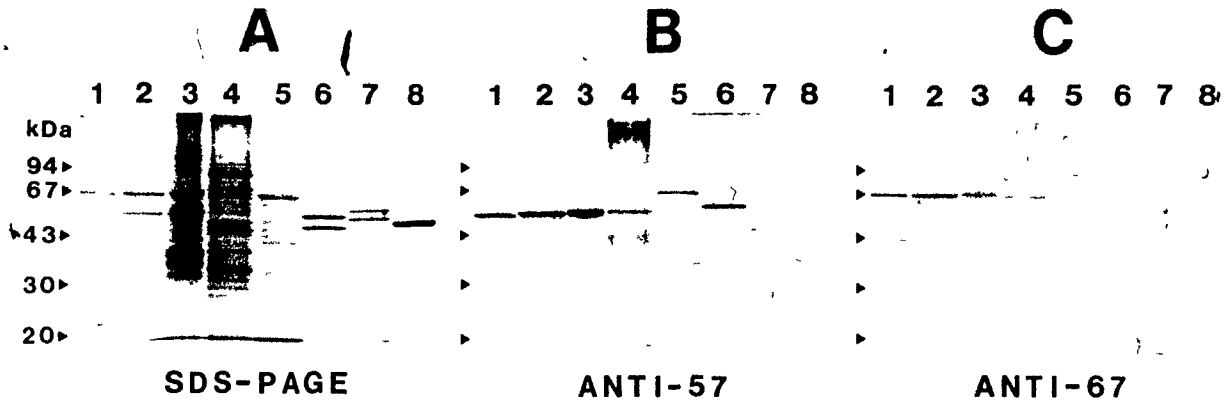
Figure 4.1 **Specificity of the anti-57 and anti-67 kDa sera for the tonoplast ATPase.** Soluble proteins (lane 1), mitochondria (lane 2), plasma membrane (lane 3), microsomal membranes (lane 4), tonoplast (lane 5) and partially-purified tonoplast ATPase (lane 6) were all isolated from storage tissue of red beet as previously described (Poole *et al.*, 1984; Manolson *et al.*, 1985). 10 μg protein from each fraction was subjected to SDS-PAGE and stained with Coomassie blue (panel A). 2.5 μg protein from each fraction was electrophoresed, blotted onto nitrocellulose and probed with a 1:770 dilution of anti-57 kDa serum (panel B) or with a 1:1400 dilution of anti-67 kDa serum (panel C), followed by biotinylated anti-rabbit goat IgG and streptavidin-biotinylated peroxidase.


A
B
C
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
kDa
94
67
43
30
20
14.4
SDS-PAGE
ANTI-57
ANTI-67

Cross-reactivity of the plant antibodies with SDS-denatured subunits of plant, animal, and fungal endomembrane $H^+$-ATPase, and bacterial, chloroplast, and mitochondrial $F_OF_1$ ATPases is shown in Fig. 4.2. In the case of beet tonoplast $H^+$-ATPase, clathrin-coated vesicle $H^+$-ATPase, chromaffin granule $H^+$-ATPase, and yeast vacuolar membranes, each antibody cross-reacted only with a subunit of corresponding size. The anti-57 kDa serum also cross-reacted with subunit "a" of the $H^+$-ATPase from the obligately anaerobic bacterium *Clostridium pasteurianum* and to the $\alpha$ subunit of the $F_1$ $H^+$-ATPase from *E. coli*. There is no cross-reactivity with chloroplast or mitochondrial ATPase.

The immunological cross-reactivity with various endomembrane $H^+$-ATPases (Fig. 4.2, lanes 1-4) is particularly striking since the preparations were not only from three different eukaryotic phyla, but also from different endomembrane organelles. Despite their common features referred to above, the endomembrane $H^+$-ATPases of different organelles are apparently not identical, but have been found to differ in physiological properties such as $Cl^-$ stimulation (see section 1.4.4) and $NO_3^-$ inhibition (see section 1.4.2) as well as in the ability to use GTP instead of ATP as energy source for $H^+$ transport (see section 1.4.5). Nevertheless, the cross-reactivity seen in lanes 1-4 of Fig. 4.2 is real, since each antiserum cross-reacts with the subunit of corresponding size from each system. Since there is no cross-reactivity of the anti-57 kDa antibody with the 67 kDa subunit, and *vice-versa*, each of the western blots of Fig. 4.2 serves as an effective

**Figure 4.2 Immunological cross-reactivity of antibodies against subunits of plant tonoplast $H^+$-ATPase with plant, animal and fungal endomembranes $H^+$-ATPase, and with bacterial, chloroplast and mitochondrial $F_1$ ATPases.** Lane 1: beet tonoplast $H^+$-ATPase (A: 2.0 μg, B: 1.0 μg, C: 0.2 μg). Lane 2: bovine brain clathrin-coated vesicle $H^+$-ATPase (A,B,C: 2.0 μg). Lane 3: bovine chromaffin granule $H^+$-ATPase (A,B,C: 11 μg). Lane 4: *Saccharomyces cerevisiae* vacuolar membranes (A: 17.5 μg, B,C: 35 μg). Lane 5: *C. pasteurianum* membrane EDTA extract (A,B,C: 7.6 μg). Lane 6: *E. coli* $F_1$ ATPase (A,B,C: 2.0 μg). Lane 7: spinach chloroplast $F_1$ ATPase (A,B,C: 2.0 μg). Lane 8: rat liver mitochondrial $F_1$ ATPase (A,B,C: 2.0 μg). Samples were subjected to SDS-PAGE using a mini-gel apparatus (Panel A), transferred to nitrocellulose, and probed with anti-57 kDa (Panel B) or anti-67 kDa serum (Panel C) using the same dilution factors and detection method as in figure 4.1. Endomembrane $H^+$-ATPases and *C. pasteurianum* were purified as previously described (Clark *et al.*, 1979; Manolson *et al.*, 1985; Percy *et al.*, 1985; Xie and Stone, 1986).


A
B
C
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
kDa
94
67
43
30
20
SDS-PAGE
ANTI-57
ANTI-67

control for the other, and confirms the specificity of the antibodies.

Antigenic cross-reactivity has been shown previously amongst subunits of $F_0F_1$ $H^+$-ATPases from plants, animals and bacteria (Rott and Nelson, 1981) and sequence homologies (Walker *et al.*, 1985) have confirmed the evolutionary conservation of structure in this class of enzymes. Similarly, amongst $E_1E_2$ ATPases, the $H^+$-translocating ATPase of yeast plasma membrane has been cloned and shown to have homologies with the $(Na^+/K^+)$-ATPase and $Ca^{2+}$-ATPase of animal cells and the $K^+$-ATPase of *E. coli* (Serrano *et al.*, 1986). We now show conservation of structure in a third distinct class of ion-pumping ATPases characteristic of endomembranes of plants, fungi and animals.

This result also provides evidence that the 57 kDa and 67 kDa polypeptides are genuine components of all endomembrane $H^+$-ATPases. While these two polypeptides are prominent in preparations of plant and fungal vacuolar $H^+$-ATPases (Mandala and Taiz, 1985a, Manolson *et al.*, 1985; Bowman *et al.*, 1986; Randall and Sze, 1986), the subunit composition of animal endomembrane $H^+$-ATPases is less clear. We do not, of course, rule out the possibility of additional functional subunits, whether restricted to certain endomembrane $H^+$-ATPases or present in all. It is noteworthy that clathrin-coated vesicle $H^+$-ATPase (Xie and Stone, 1986), in which the largest number of distinct polypeptides have been reported, is the only case in which the purified enzyme has been reconstituted in liposomes and shown to transport $H^+$ as well as to hydrolyse ATP.

Although the antibodies also cross-react with subunits of bacterial $F_0F_1$ ATPases (Fig. 4.2, lanes 5,6), correspondence in subunit size is no longer seen. In order to judge more accurately the intensity of this cross-reaction, western blots were probed with $^{125}$I-protein A, and autoradiographs were compared by densitometry with the intensity of Coomassie blue staining of the gel. For the cross-reaction of the anti-57 kDa antibody with subunit "a" of the $H^+$-ATPase from *C. pasteurianum* and to the $\alpha$ subunit of the *E. coli* $H^+$-ATPase, the extent of antibody binding per unit protein was in the same range as for the animal and fungal endomembrane $H^+$-ATPases (data not shown). Cross-reactivity with the bacterial $H^+$-ATPases, with affinity to the *E. coli* $H^+$-ATPase and to the simpler $H^+$-ATPase (Clark *et al.*, 1979) of the more primitive organism (Schwartz and Dayhoff, 1978), suggests a common origin for endomembrane $H^+$-ATPases and $F_1F_0$ ATPases. Lack of cross-reactivity with chloroplast and mitochondrial ATPases further suggests that these more highly specialized ATPases may have diverged to a greater extent than the endomembrane $H^+$-ATPases from the ancestral type. We await protein/nucleic acid sequence data to confirm these ideas.

# Chapter 5. SEQUENCE AND ANALYSIS OF A cDNA ENCODING A NUCLEOTIDE-BINDING SUBUNIT OF THE TONOPLAST ATPase FROM *ARABIDOPSIS*

## 5.1 ABSTRACT

Functional and structural similarities among a wide variety of endomembrane $H^+$-ATPases suggest that they form a distinct class with a common origin. Immunological studies (Chapter 4) support this idea and suggest an evolutionary relationship between the endomembrane and $F_OF_1$ ATPases. Further examination of relationships necessitates comparison of protein/nucleic acid sequence data. To this end, we have cloned and sequenced the cDNA encoding the 57 kDa polypeptide of the *Arabidopsis* vacuolar membrane $H^+$-ATPase.

This cDNA encodes a hydrophilic polypeptide containing a putative ATP binding site. Lack of a secretion signal sequence suggests it is not processed through the endoplasmic reticulum but translated on cytosolic ribosomes. Comparison of protein sequences shows the 57 kDa subunit from *Arabidopsis* to be nearly identical with the corresponding subunit in *Neurospora* vacuolar membrane $H^+$-ATPase, very similar to the $\beta$ subunit of the archaebacterium *Sulfolobus*, and slightly, but nevertheless significantly, homologous to the $\alpha$ and $\beta$ subunits of the $F_OF_1$ ATPases. These results suggest that these different classes of ATPases have evolved from a common ancestor.

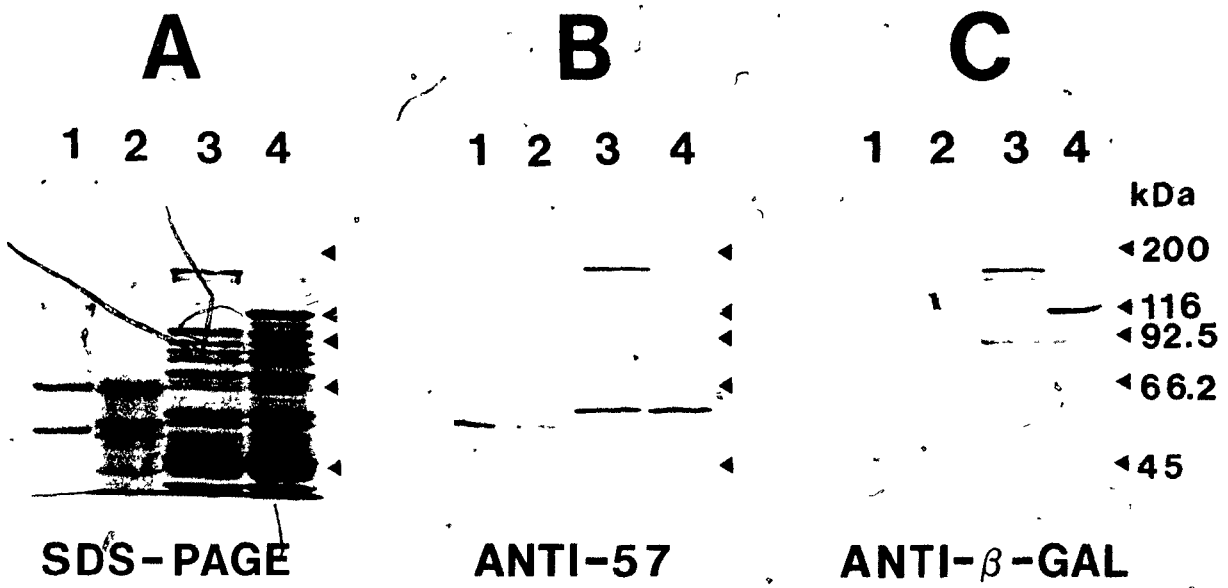
## 5.2 RESULTS

### 5.2.1 Selection and Characterization of a Clone from a λgt11 Expression Library

Western blots were used to test the practicality of screening an expression library from *Arabidopsis* with antibodies raised against a polypeptide from red beet. Figure 5.1 shows beet tonoplast $H^+$-ATPase and microsomal pellet proteins from *Arabidopsis* plants subjected to SDS-PAGE (panel A, lanes 1,2), transferred to nitrocellulose and probed with the anti-57 kDa antibody (panel B, lanes 1,2). Immunodetection shows only one polypeptide from *Arabidopsis* migrating alongside the 57 kDa polypeptide of beet tonoplast $H^+$-ATPase.

600,000 pfu from an *Arabidopsis* leaf cDNA λgt11 library were screened with the anti-57 kDa antibody, resulting in one positive. The positive clone contained a 1.9 kb insert and produced an IPTG-inducible fusion protein of about 170,000 kDa as determined by SDS-PAGE (Fig. 5.1, panel A, lane 3). The β-galactosidase portion of the fusion protein accounts for 114 kDa, (Huynh *et al.*) implying the insert codes for a 56 kDa polypeptide. The fusion protein is recognized both by anti-57 antibodies (Fig. 5.1, panel B, lane 3) and anti-β-galactosidase antibodies (Fig. 5.1, panel C, lane 3). Wild type λgt11 β-galactosidase is not detected by anti-57 serum (Fig. 5.1, panel B, lane 4) nor is the 57 kDa polypeptide of the tonoplast $H^+$-ATPase detected by anti-β-galactosidase serum (Fig. 5.1, panel C, lanes 1,2). A 60 kDa polypeptide recognized by the anti-57

**Figure 5.1 Characterization of β-galactosidase / 57 kDa fusion protein.** Lane 1: beet tonoplast $H^+$-ATPase (A: 2.0 μg, B,C: 0.2 μg). Lane 2: *Arabidopsis* microsomal pellet (A,B,C: 5 μg). Lane 3: crude lysate from λgt11 recombinant lysogen containing the fusion protein (A: 18 μg, B,C: 3.5 μg). Lane 4: crude lysate from wild type λgt11 recombinant lysogen (A: 18 μg, B,C: 3.5 μg). Samples were subjected to SDS-PAGE (10% acrylamide) using a mini-gel apparatus (panel A), transferred to nitrocellulose, and probed with a 1:750 dilution of anti-57 serum (panel B) or a 1:1000 dilution of anti-β-galactosidase serum (panel C).

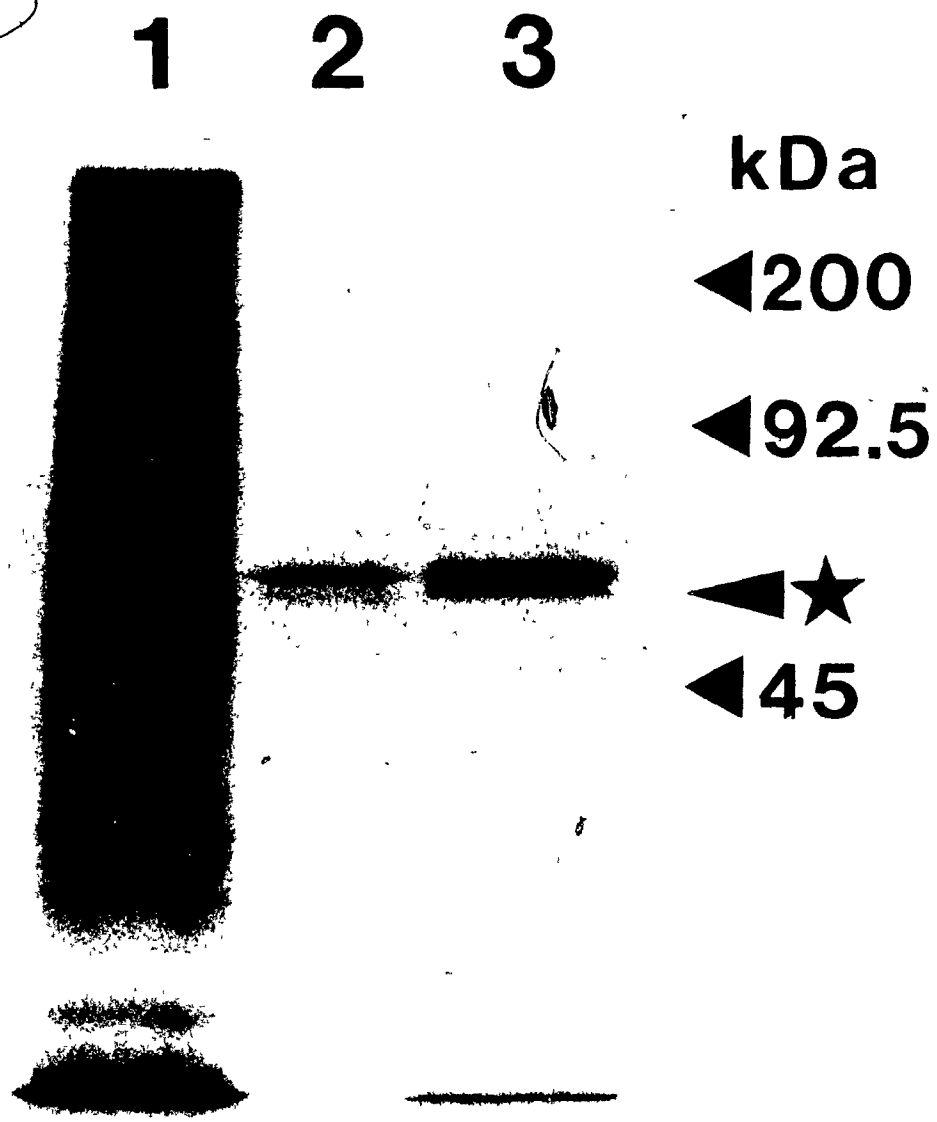

A
B
C
1 2 3 4
1 2 3 4
1 2 3 4
kDa
200
116
92.5
66.2
45
SDS-PAGE
ANTI-57
ANTI-β-GAL

sera in the *E. coli* cell lysates (Fig. 5.1, panel B, lanes 3,4) is the α subunit of the bacterial $F_1$-ATPase. (see chapter 3).

5.2.2 *In Vitro* Transcription and *In Vitro* Translation of Insert cDNA from p57kDa

The 1.9 kb insert obtained above was subcloned into the unique EcoRI site of KS M13$^+$ Bluescript (referred to below as p57kDa). *In vitro* transcription of the insert cDNA followed by *in vitro* translation of the resulting RNA produced two polypeptides, of 57 and 59 kDa respectively (Fig. 5.2, lane 3). Poly(A)$^+$ RNA isolated from whole *Arabidopsis* plants was *in vitro* translated (Fig. 5.2, lane 1) and immunoprecipitated with anti-57 kDa sera (Fig. 5.2, lane 2). Two polypeptides were seen of identical size to the ones produced by the *in vitro* transcribed-*in vitro* translated p57kDa insert. There were no labeled polypeptides visualized when immunoprecipitation was performed with the same dilution of non-immune serum (data not shown). The smaller of the two polypeptides migrated alongside the 57 kDa polypeptide of the red beet tonoplast H$^+$-ATPase (identified by a star in Fig. 5.2). The *in vitro* translation of two polypeptides, rather than the expected one, may result from the use of a heterologous *in vitro* translation system (see Discussion).

Figure 5.2 **Characterization of coding regions of insert cDNA from p57kDa.** Lane 1: *in vitro* translation of Poly(A)$^+$ RNA isolated from whole *Arabidopsis* plants. Lane 2: immunoprecipitation of *in vitro* translated products shown in lane 1 with a 1:750 dilution of anti-57 kDa serum. Lane 3: *in vitro* translation of *in vitro* transcribed insert cDNA from p57kDa. Samples were subjected to SDS-PAGE (10% acrylamide) on a mini-gel apparatus. $^{35}S$ label was visualized fluorographically. The star indicates the position of the 57 kDa polypeptide of the beet tonoplast $H^+$-ATPase on SDS-PAGE.


1
2
3
kDa
200
92.5
★
45

### 5.2.3 Primer Extension

Primer extension of total *Arabidopsis* RNA containing an oligonucleotide complementary to a region 50 bp downstream from the 5′ end of p57kDa's insert produced one 162 bp product (Fig. 5.3, lane 1). This result implies that the p57kDa insert is missing 112 bp, but from Fig. 5.2 it seems that these are non-coding sequences in the 5′ end.

### 5.2.4 Nucleotide Sequence

The strategy used to sequence both strands of the p57kDa insert is shown in Fig. 5.4. The sequence of the p57kDa insert is shown in Fig. 5.5. There are three putative start codons at position 112, 130 and 178 (Fig. 5.5). The flanking sequences of the second methionine at position 130 (AAC ATG G) best matches the consensus sequence for plant initiation codons (ANN ATG G) reported by Heidecker and Messing (1986). Kozak (1981) showed that wheat germ ribosomes bound most strongly to start codons with a G at position +1. Lütcke *et al.* (1987) demonstrated that the A in position -3, while critical with reticulocyte lysates, had no effect on translational efficiencies when using wheatgerm systems. This evidence suggests the third methionine at position 178 (GGC ATG G) may be a possible plant initiation site. The G at the -3 position is the second most frequently used nucleotide (18%) in that position (Kozak, 1984). Using Staden's weight matrix method for ribosome binding site location (Staden, 1984), which is based on the assumption of base pairing between mRNA and the 3′ region of 18S rRNA (Sargan *et al.*, 1982), both the second and

Figure 5.3 **Size determination of RNA coding for the 57 kDa polypeptide by primer extension.** $^{32}P$ 5′ end-labeled 21 bp oligonucleotide, complementary to a region 50 bp downstream from the 5′ end of the cloned insert of p57kDa was annealed to *Arabidopsis* RNA, followed by reverse transcription by primer extension. The primer-extended product was run on a 5% sequencing gel (lane 1). For comparison a sequencing ladder obtained using the same primer is run on lane 2. The arrow points to the EcoRI site where the 5′ end of the cloned insert has been ligated into Bluescript.

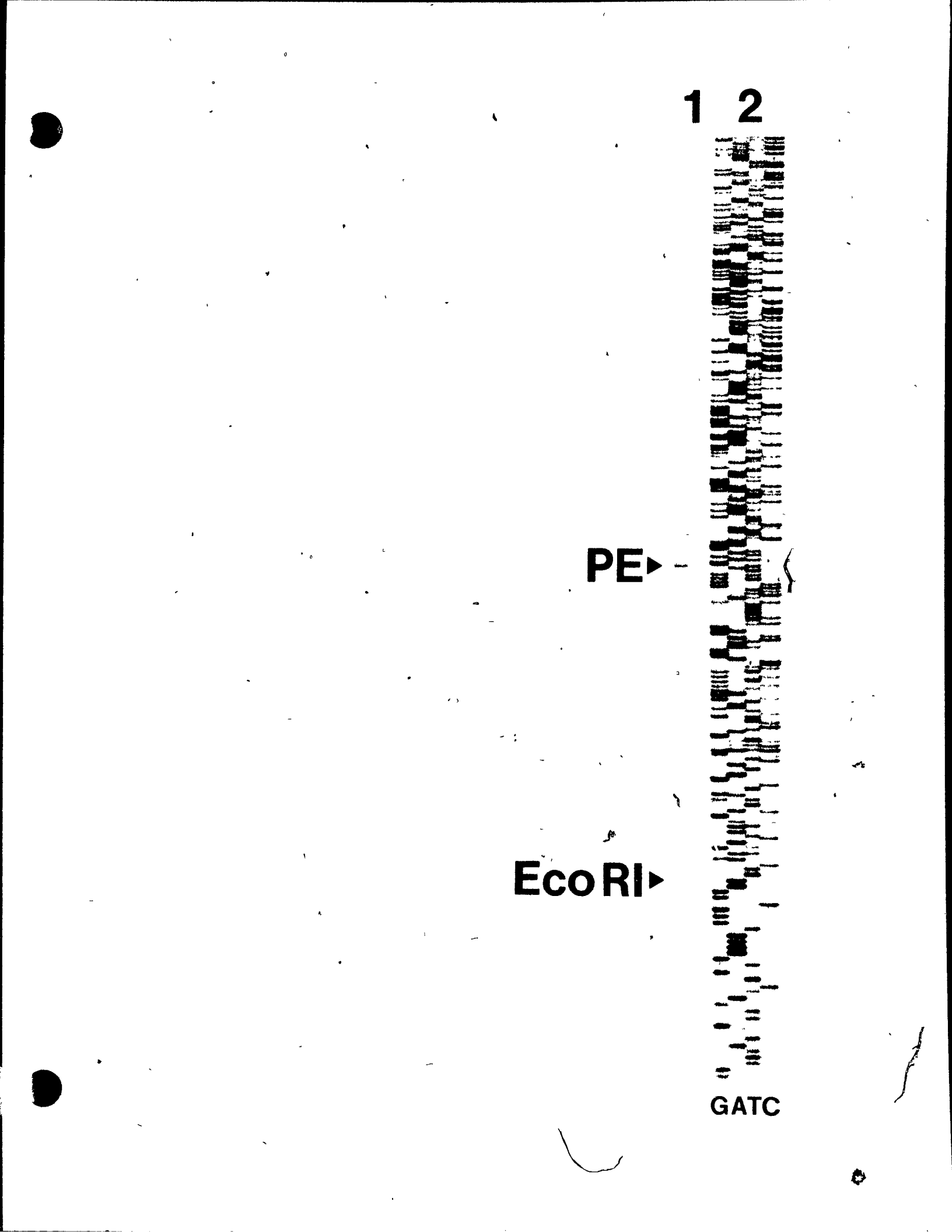

1 2
PE
EcoRI
GATC

**Figure 5.4 Sequencing strategy of insert cDNA from p57kDa.** Insert cDNA from p57kDa is represented by the solid thin line, Bluescript by the open ended thick hollow bars and restriction enzyme cleavage sites by the short vertical lines. A series of deletions on p57kDa were made using EcoRI and XbaI, subcloning the restriction fragments in both orientations into the Bluescript polylinker. These clones were sequenced using the T7 or SK primers (represented by the solid arrows). Synthesized oligonucleotides were then used as primers to sequence the remainder (dashed arrows) such that both stands were sequenced to completion.

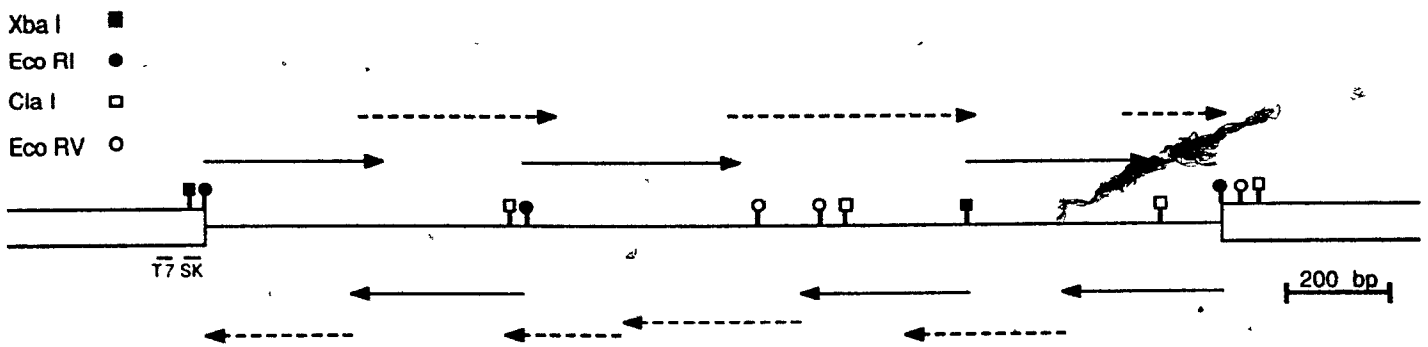

Xba I
Eco RI
Cla I
Eco RV
T7
SK
200 bp

Figure 5.5 **Nucleotide sequence and predicted amino acid sequence of insert cDNA from p57kDa.** The three possible initiation sites are indicated by capitalizing the corresponding methionines. The putative ATP binding site is underlined by a solid line. The two potential polyadenylation signals are underlined by a dashed line. The Stop codon is represented by ***.

```
1     CGCCCATTTTCACAGATCAACGATAAACCAAATCAAAGCAGCTCCTGGGAACGGTTCGAT

61    TCGAGCAGAGAGAGAGGGAGAGAGGGAGAGAGGGAGAGAGAGTTATGGTTTATGAGTCTC
                                                    METSerLeu

121   TCTGTGAACATGGGGACGAATGATCTCGACATCGAAGAAGGGACTCTGGAGATCGGCATC
      SerValAsnMETGlyThrAsnAspLeuAspIleGluGluGlyThrLeuGluIleGlyMET

181   GAGTATAGAACTGTTTCTGGTGTTGCTGGACCATTGGTCATTCTTGACAAAGTGAAGGGT
      GluTyrArgThrValSerGlyValAlaGlyProLeuValIleLeuAspLysValLysGly

241   CCAAAGTACCAGGAGATTGTTAATATTCGCTTAGGAGATGGATCAACGAGACGTGGTCAG
      ProLysTyrGlnGluIleValAsnIleArgLeuGlyAspGlySerThrArgArgGlyGln

301   GTTTTGGAAGTTGATGGCGAGAAAGCAGTTGTGCAGGTTTTTGAAGGAACATCTGGAATT
      ValLeuGluValAspGlyGluLysAlaValValGlnValPheGluGlyThrSerGlyIle

361   GACAACAAGTTTACAACCGTGCAATTCACAGGAGAGGTTTTGAAAACACCTGTATCATTG
      AspAsnLysPheThrThrValGlnPheThrGlyGluValLeuLysThrProValSerLeu

421   GACATGCTTGGGCGCATATTTAACGGTTCAGGAAAGCCGATTGATAATGGCCCTCCTATT
      AspMetLeuGlyArgIlePheAsnGlySerGlyLysProIleAspAsnGlyProProIle

481   CTGCCAGAAGCATACCTTGATATTTCAGGAAGTTCAATCAACCCCAGTGAAAGAACCTAT
      LeuProGluAlaTyrLeuAspIleSerGlySerSerIleAsnProSerGluArgThrTyr

541   CCTGAAGAGATGATACAGACAGGCATATCGACCATCGATGTCATGAATTCCATTGCTCGT
      ProGluGluMetIleGlnThrGlyIleSerThrIleAspValMetAsnSerIleAlaArg

601   GGACAGAAGATTCCACTTTTCTCTGCTGCTGGTCTTCCACATAATGAAATAGCTGCTCAG
      GlyGlnLysIleProLeuPheSerAlaAlaGlyLeuProHisAsnGluIleAlaAlaGln

661   ATTTGTCGTCAGGCTGGTCTAGTCAAGCGTTTGGAAAAGACTGTTGATCTACTTGAGGAT
      IleCysArgGlnAlaGlyLeuValLysArgLeuGluLysThrValAspLeuLeuGluAsp

721   CATGGAGAGGACAATTTTGCAATTGTGTTTGCAGCTATGGGTGTGAACATGGAGACAGCT
      HisGlyGluAspAsnPheAlaIleValPheAlaAlaMetGlyValAsnMetGluThrAla

781   GAGTTCTTCAAGCGAGATTTTGAAGAAAATGGATCAATGGAGAGAGTTACTCTTTTCCTG
      GlnPhePheLysArgAspPheGluGluAsnGlySerMetGluArgValThrLeuPheLeu

841   AACCTGGCCAATGACCCAACCATTGAGAGAATCATCACTCCTCGAATTGCCCTCACAACA
      AsnLeuAlaAsnAspProThrIleGluArgIleIleThrProArgIleAlaLeuThrThr

901   GCTGAATATCTGGCTTATGAATGTGGGAAACACGTCCTTGTGATATTGACGGATATGAGT
      AlaGluTyrLeuAlaTyrGluCysGlyLysHisValLeuValIleLeuThrAspMetSer

961   TCTTATGCTGATGCTCTTCGTGAGGTTTCCGCTGCCCGAGAAGAGGTTCCCCGAAGACGT
      SerTyrAlaAspAlaLeuArgGluValSerAlaAlaArgGluGluValProGlyArgArg

1021  GGATATCCAGGTTATATGTACACTGATCTTGCAACTATTTATGAACGTGCTGGGCGTATA
      GlyTyrProGlyTyrMetTyrThrAspLeuAlaThrIleTyrGluArgAlaGlyArgIle

1081  GAAGGAAGAAAAGGTTCCATCACCCAAATTCCAATCCTCACTATGCCCAATGACGATATC
      GluGlyArgLysGlySerIleThrGlnIleProIleLeuThrMetProAsnAspAspIle

1141  ACTCATCCAACTCCGGATCTTACTGGTTACATTACTGAAGGTCAGATATATATCGATAGG
      ThrHisProThrProAspLeuThrGlyTyrIleThrGluGlyGlnIleTyrIleAspArg

1201  CAACTTCACAACAGACAGATATATCCACCCATCAACGTGCTTCCATCCCTTTCTCGTTTA
      GlnLeuHisAsnArgGlnIleTyrProProIleAsnValLeuProSerLeuSerArgLeu

1261  ATGAAGAGTGCTATCGGCGAGGGCATGACTCGTAAAGACCATTCTGATGTGTCGAACCAG
      MetLysSerAlaIleGlyGluGlyMetThrArgLysAspHisSerAspValSerAsnGln

1321  CTGTATGCAAATTATGCAATCGGGAAAGATGTTCAAGCGATGAAAGCTGTTGTTGGAGAA
      LeuTyrAlaAsnTyrAlaIleGlyLysAspValGlnAlaMetLysAlaValValGlyGlu

1381  GAAGCACTTTCTTCAGAGGATTTGCTTTATCTAGAGTTTTTGGATAAGTTTGAGAGGAAG
      GluAlaLeuSerSerGluAspLeuLeuTyrLeuGluPheLeuAspLysPheGluArgLys

1441  TTTGTGATGCAAGGAGCTTATGATACACGCAACATCTTCCAGTCGCTGGACTTAGCTTGG
      PheValMetGlnGlyAlaTyrAspThrArgAsnIlePheGlnSerLeuAspLeuAlaTrp

1501  ACATTGCTCCGTATCTTCCCACGGGAGCTTCTTCATCGTATCCCTGCAAAGACACTTGAC
      ThrLeuLeuArgIlePheProArgGluLeuLeuHisArgIleProAlaLysThrLeuAsp

1561  CAATTCTACAGCCGCGACTCAACCAGTTAAAATGAGGTAATGGAGGTTATCTTATCGAAA
      GlnPheTyrSerArgAspSerThrSer***

1621  CTCTTTTGAGAGAAAAGTGTGAATTTTTGTGATGTGTATTATTTGGTGCTTATTATAAAG

1681  AGGAAAAACAAAGAAAAGCTATATATTCTGTGTCTCCCTATCTGGTGATATTTTTTTTGG

1741  TTCTGCATTGTGTTCCAAAGTGGAAATAAAAATCGATAAACGATGTCGTATTGTAGTACT

1801  TTCCTTTTCTTTGTATGAATTTGTTAAGATTGGTTTATAAATGGGATTATAATAAGTATC

1861  TTTAAAAAAAAAAAAAAA
```

third methionines are calculated to be possible eukaryotic binding sites, but with a higher probability computed for the second methionine.

After the first ATG there is a 1476-nucleotide open reading frame followed by 135 nucleotides of 3' non-coding region. Two putative polyadenylation signal sequences were found 30 nucleotides upstream from the poly $(A)^+$ tail (Fig. 5.5), both in close agreement with the plant consensus sequence of (A/T)AATAA(A/G) reported by Heidecker and Messing (1986).

### 5.2.5 Amino Acid Sequence

The open reading frame of the p57kDa insert codes for a 492 amino acid polypeptide with a predicted molecular weight of 55 kDa. The difference calculated between the putative start codons is 0.6 kDa (6 amino acids) between the first and second methionine and 1.7 kDa (16 amino acids) between the second and third methionine. The highest predicted value is still 2 kDa lower than the expected 57 kDa calculated by SDS-PAGE. The predicted pI values for polypeptides starting at the first, second and third AUG are 4.74, 4.75 and 5.0 respectively. The calculated pI value of 5.0 is the closest to the pI values obtained for this subunit by isoelectric focusing: 5.2 with red beet tissue (Dupont *et al.* 1988), and 5.1 with zucchini (unpublished work in collaboration with Georg Martiny-Baron).

The hydrophobicity of the predicted polypeptide was examined using four different methods. Hydrophobicity plots were calculated using the method of Kyte and Doolittle (1982) with windows ranging from 5 to 15 amino acids with no large

uninterrupted areas appearing above the midpoint line of -0.4. The midpoint line of -0.4 represents the mean of the grand average of hydropathy value (GRAVY) for sequenced soluble proteins, hydrophobic values lying above and hydrophilic values lying below the midpoint line. The calculated GRAVY score of -2.28 suggest that the predicted 57 kDa polypeptide is a soluble protein. Membrane-associated helices were not predicted with either the algorithm of Eisenberg *et al.* (1984) or that of Rao and Argos (1986) using the parameters and cut-off points proposed in the original articles. The computer program written by Klein *et al.* (1984) placed the odds at 27 to 1 for the predicted sequence to be a peripheral as opposed to an integral protein.

Heijne's SRP signal sequence-detecting algorithm (Heijne, 1986) could not detect any region near the N-terminal that scored higher than 3.5. The value of 3.5 was used as a cut off because 98% of published signal sequences scored above it and 98% of published cytosolic sequences scored below it.

### 5.2.6 Protein Homologies

Homologies with the predicted structure of the 57 kDa polypeptide were examined using Lipman and Pearson's protein similarity searching algorithm FASTP (Lipman and Pearson, 1985), with a single amino acid look-up table (ktup =1), to search the National Biomedical Research Foundation protein sequence database (PIR) release 12. Evaluation of statistical significance was made by comparing the query sequence to 50 randomly-permutated versions of the putative related sequence

and calculating the Z value as described by Lipman and Pearson (1985). Z values above 10 were considered by the authors as significant homologies, while values over 3 were judged as possibly significant. Table 5.1 lists $H^+$-ATPases and ATP-binding proteins in decreasing order of their Z values. The table includes proteins from the above database plus unpublished data of Emma Jean Bowman, Barry Bowman, Lincoln Taiz and Masasuke Yoshida. A Z value of 299.3 for the 57 kDa polypeptide ($\beta$ subunit) of *Neurospora* vacuolar $H^+$-ATPase reflects that in a 465 amino acid overlap, 76% of the sequence is identical to the putative *Arabidopsis* $\beta$ subunit. The $\beta$ subunit of the ATPase of *Sulfolobus* had the second highest score of 137.7, calculated from 53% identity in a 470 amino acid overlap. The next eleven Z scores (ranging between 10 and 20) were the $\alpha$ and $\beta$ subunits of the $F_0F_1$ $H^+$-ATPase and the $\alpha$ subunits of endomembrane $H^+$-ATPases. For comparison, the top scores for other ATP-binding proteins are listed, although none of them had Z scores over 3.

Table 5.2 shows the region which is the most conserved over the greatest number of $H^+$-ATPase subunits. The first column of amino acids is the most variable with both positive and negatively charged residues. The four non-conserved amino acids found in the fourth column are all conservative changes from isoleucine to other non-polar residues. In the $\beta$ subunit of the bovine mitochondrial $H^+$-ATPase, lysine 301 and isoleucine 304 (underlined in Table 5.2) have been shown to bind azido-ATP, suggesting this region is part of a putative ATP binding site (Hollemans *et al.* 1983).

Table 5.1 **Protein Sequence Similarities with the 57 kDa ($\beta$) Subunit of the Endomembrane $H^+$-ATPase from *Arabidopsis***

| Subunit | z value |
|---|---|
| **ATPases** ( z > 100 ) | |
| 57 kda ($\beta$), *Arabidopsis* vacuolar membrane | 329.5 (optimal score) |
| 57 kDa ($\beta$), *Neurospora* vacuolar membrane | 299.3 |
| $\beta$, *Sulfolobus* plasma membrane | 137.7 |
| **ATPases** ( z > 10 ) | |
| $\beta$, $F_OF_1$ bovine mitochondrion | 18.6 |
| $\beta$, $F_OF_1$ tobacco chloroplast | 17.2 |
| $\beta$, $F_OF_1$ maize chloroplast | 16.1 |
| $\alpha$, $F_OF_1$ *E. coli* | 15.6 |
| $\beta$, $F_OF_1$ spinach chloroplast | 15.0 |
| $\beta$, $F_OF_1$ *E. coli* | 14.8 |
| $\beta$, $F_OF_1$ barley mitochondrion | 14.4 |
| $\beta$, $F_OF_1$ barley chloroplast | 13.9 |
| 70 kDa ($\alpha$) *Neurospora* vacuolar membrane | 12.8 |
| 69 kDa ($\alpha$) maize vacuolar membrane | 11.5 |
| $\alpha$, $F_OF_1$ tobacco chloroplast | 11.5 |
| **Other ATP-binding Proteins** ( z < 2 ) | |
| ATP phosphoribosyltransferase | 1.8 |
| Myelin basic protein | 1.1 |
| Adenylate kinase | 1.0 |
| $Ca^{2+}$ ATPase | 0.8 |
| ADP,ATP carrier protein | 0.8 |

Table 5.2 **Putative ATP Binding Site**

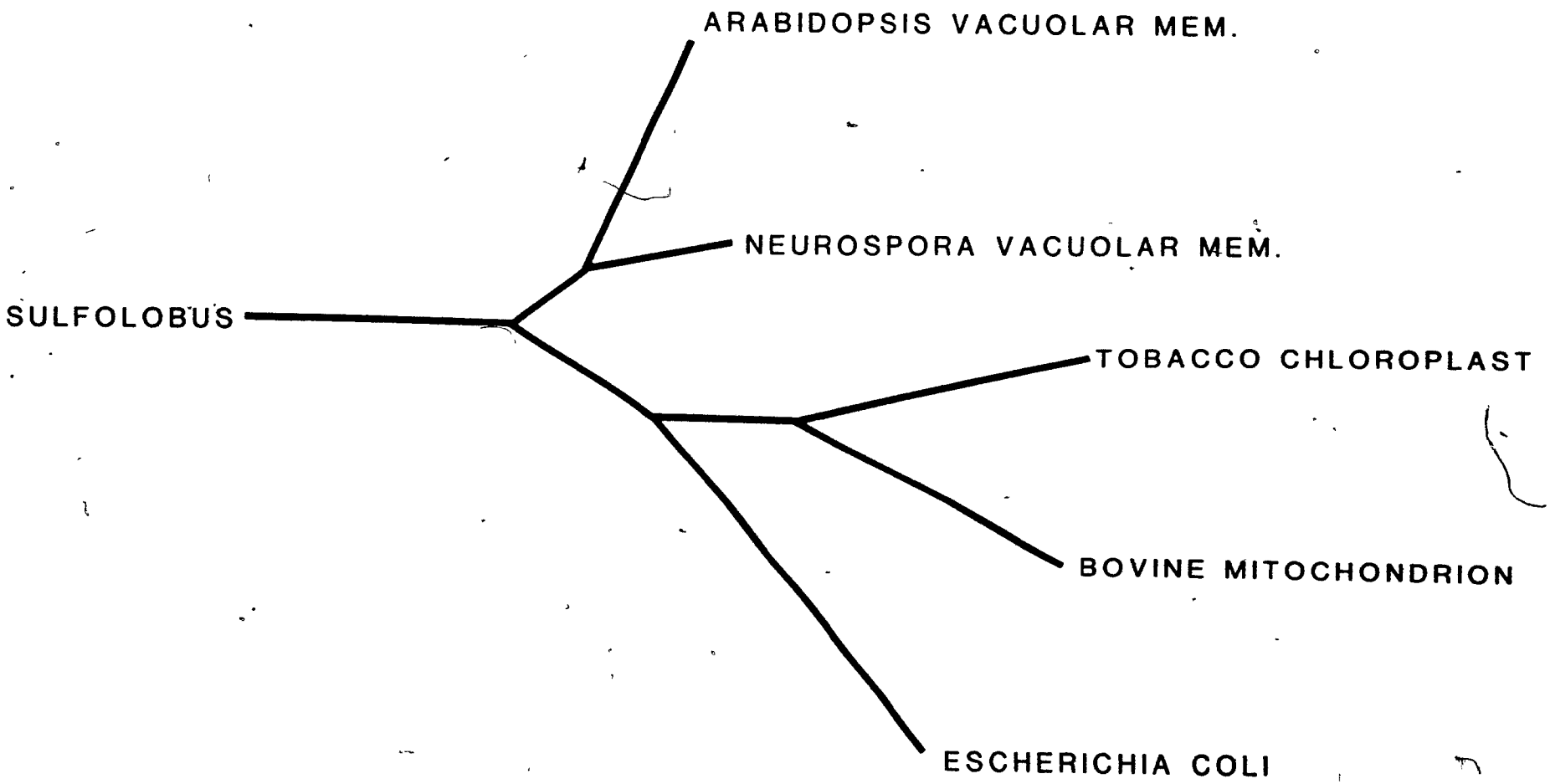
| ATPase subunit | |
|---|---|
| 57 kDA, *Arabidopsis* vacuolar membrane | 328-LYS-GLY-SER-ILE-THR |
| 60 kDa, *Neurospora* vacuolar membrane | 321-ASN-GLY-SER-ILE-THR |
| 69 kDa, carrot vacuolar membrane | 404-ASN-GLY-SER-VAL-THR |
| $\beta$, Sulfolobus | 229-LYS-GLY-SER-ILE-THR |
| $\beta$, barley chloroplast | 318-LYS-GLY-SER-ILE-THR |
| $\beta$, maize chloroplast | 319-LYS-GLY-SER-ILE-THR |
| $\beta$, spinach chloroplast | 319-GLU-GLY-SER-ILE-THR |
| $\beta$, tobacco chloroplast | 319-GLU-GLY-SER-ILE-THR |
| $\beta$, bovine mitochondrion | 301-LYS-GLY-SER-ILE-THR |
| $\beta$, barley mitochondrion | 139-LYS-GLY-SER-VAL-THR |
| $\alpha$, *E. coli* | 322-THR-GLY-SER-LEU-THR |
| $\alpha$, tobacco chloroplast | 312-GLU-GLY-SER-MET-THR |

Consensus Sequence: LYS(50%)-GLY(100%)-SER(100%)-ILE(68%)-THR(100%)

5.2.7 Phylogeny

Fig 5.6 shows an unrooted phylogeny inferred from the protein sequences of the most similar subunits of six different $H^+$-ATPases using Felsenstein's "Protein Sequence Parsimony Method" (PROTPARS) version 3.0. PROTPARS's algorithm calculates the number of steps required for changes in amino acids, consistent with the genetic code, with the assumption that the data entered is already correctly aligned. Considering that parsimony methods tend to fail when dealing with data involving large amounts of change (Felsenstein, 1981), to remain within the confidence of the method only the six most homologous $H^+$-ATPase subunit sequences (as compared to the $\beta$ subunit of the *Arabidopsis* tonoplast $H^+$-ATPase) were used. Alignment of the sequences was done manually with the aid of FASTP. Deletions were added to the sequences in order to compensate for differences in length and to align highly conserved regions. The program was run 5 times with the global rearrangement option, the order of sequences being randomly shuffled each time. Regardless of the order of data, the phylogenetic tree in Fig. 5.6 was produced each time. In order to facilitate the calculations, the assumption was made that any amino acid change to any other one should count as one step (Eck and Dayhoff, 1966) for the scale of the branches, although this will underestimate the length.

Figure 5.6 **Unrooted phylogenetic tree of $H^+$-ATPase subunits.** The program PROTPARS was used to infer phylogeny amongst the following $H^+$-ATPase subunits. (1) 57 kDa ($\beta$) subunit of *Arabidopsis* tonoplast $H^+$-ATPase, (2) 57 kDa ($\beta$) subunit of *Neurospora* vacuolar membrane $H^+$-ATPase, (3) $\beta$ subunit of *Sulfolobus* plasma membrane $H^+$-ATPase, (4) $\alpha$ subunit of *E. coli* $F_0F_1$ $H^+$-ATPase, (5) $\beta$ subunit of tobacco chloroplast $F_0F_1$ $H^+$-ATPase, (6) $\beta$ subunit of bovine mitochondrial $F_0F_1$ $H^+$-ATPase.


ARABIDOPSIS VACUOLAR MEM.
NEUROSPORA VACUOLAR MEM.
SULFOLOBUS
TOBACCO CHLOROPLAST
BOVINE MITOCHONDRION
ESCHERICHIA COLI

## 5.3 DISCUSSION

The cDNA encoding the 57 kDa polypeptide of the *Arabidopsis* tonoplast ATPase was selected from an *Arabidopsis* leaf cDNA λgt11 expression library. The initial evidence of the clone's identity was from the fusion protein's strong antigenic response to the anti-57 kDa antibodies (Fig. 5.1) and from the estimation of polypeptide size (Fig. 5.2). The high degree of protein sequence homology with a range of $H^+$-ATPase subunits and the near identity with the 57 kDa polypeptide of the *Neurospora* vacuolar $H^+$-ATPase (Table 5.1) confirmed its identity.

The insert cDNA from p57kDa contained the entire coding region for the 57 kDa polypeptide (Fig. 5.2). The sequence included the 3' non-coding region since it ends with a poly $^+$(A) tail (Fig. 5.5), but is missing 112 bp of non-coding sequence on the 5' end (Fig. 5.3).

The presence of three closely-spaced methionines (capitalized in Fig. 5.5) at the beginning of the coding region obscures the estimation of where initiation starts. The flanking region surrounding the first AUG does not match any published consensus sequence for initiation sites, yet Von Heijne (1987) claims that even in the absence of consensus signals 90% of start sites begin with the most 5' AUG. The flanking regions of the second and third AUGs suggest that either could be plant initiation sites, but with a higher probability for the former. Considering a mammalian

translation system dealing with plant signals, two possible initiation sites may explain the two polypeptides in the *in vitro* translated products (Fig. 5.2, lanes 2 and 3). The 2.3 kDa difference between the first and third, or the 1.7 kDa difference between the second and third methionine could account for the approximately 2 kDa difference between the two *in vitro* translated polypeptides. The 57 kDa polypeptide of beet tonoplast $H^+$-ATPase co-migrated with the lower of the two polypeptides (Fig. 5.2) suggesting that *in vivo* initiation starts on the third AUG. Initiation starting on the third AUG would result in a pI value close to values obtained by isoelectric focusing, but a mol. wt. further away from SDS-PAGE estimations. Differences between predicted and SDS-PAGE-calculated molecular weights could be due to anomalous amounts of SDS being bound to the protein, resulting in exposure of some charged amino acid, or of irregularities in the shape of the SDS-protein complex (Maddy, 1976).

Predictions based on the amino acid sequence of the cloned cDNA agree with biochemical data. Four different computer programs predicted the encoded polypeptide to be a soluble protein. These predictions concur with the results of Rea *et al.* (1987a) showing the corresponding subunit in red beet can be dissociated from the vacuolar membrane by treatment with chaotropic anions. The absence of any detectable SRP signal sequence is in concordance with evidence showing that the same subunit in corn is synthesized on cytosolic ribosomes (Steven Randall and Heven Sze, personal communication). The presence of the putative ATP binding site in the sequence (Table 5.2) is

in accord with photoaffinity observations (Chapter 3) that Bz-ATP binds to the same subunit in red beet.

Statistically significant homologies (Z values greater than 10) between the $\alpha$ and $\beta$ subunits of $F_0F_1$ $H^+$-ATPase and the predicted 57 kDa subunit in *Arabidopsis* are shown in Table 5.1. Considering structural and functional similarities, sequence homologies are expected. The subunits are all part of large multimeric $H^+$-translocating, ATP-hydrolyzing enzymes, have ATP binding sites, are only peripherally bound to their respective membranes and are similar in size. It appears that sequence homologies do not just reflect functional similarities, since other ATP binding and ATP hydrolyzing enzymes (ATP phosphoribosyltransferase, myelin, adenylate kinase, $Ca^{2+}$ ATPase, ADP,ATP carrier protein) show no significant degree of homology. This suggests that the homologies may also reflect a common evolutionary origin. The limits of similarities (all Z values are less than 20 for $F_0F_1$ ATPases) may in part reflect differences in function as well as evolutionary distance.

Deducing phylogenetic relationships on the basis of sequence homologies assumes a constant function. Functional changes in an enzyme bring additional changes in sequence which obscure evolutionary distance (Woese, 1981). The endomembrane ATPases hydrolyse ATP and pump protons into the inside of their respective organelles. The generated proton gradient is in turn used for secondary transport. In contrast, the $F_0F_1$ ATPases generally synthesize ATP using a proton gradient generated by oxidative or photosynthetic electron transport. The role of the *Sulfolobus* ATPase has yet to be demonstrated.

By definition, enzymatic reactions are reversible, but structural changes will affect in which direction the reaction is kinetically favored. Thus part of the dissimilarity amongst subunit sequences may reflect change in function as opposed to evolutionary distance from a common origin. This is not to argue that these ATPases are not suitable for phylogenetic analysis, but to point out that the evolutionary distances calculated by sequence comparison may be overestimated.

To explore the evolutionary aspects of the sequence homologies, a phylogenetic tree was calculated using the protein sequences of six ATPase subunits (Fig. 5.6). The validity of the results are dependent upon the subjective task of aligning the sequences. For this reason the six subunits with the most apparent alignments were chosen. Although it would be most appropriate to use the corresponding subunits from each type of ATPase, the question of which subunit corresponds to which is still under debate (see Chapter 1). As it happens, this issue has little effect on the form of the tree. The PROTPARS program was rerun except for substituting the $\alpha$ subunits for the $\beta$ subunits in the chloroplast and mitochondrial ATPases and switching the $\beta$ for the $\alpha$ subunit in E. coli ATPase. The only difference to the tree was an increase in the number of steps between the branches reflecting the necessity of adding more deletions to compensate for different sizes.

The validity of the tree may be examined by discussing its components. Fig. 5.6 shows the mitochondrial and chloroplast subunits arise from the same node, which in turn is connected

to the branch leading to the *E. coli* subunit. This pattern is in agreement with the evolutionary tree constructed by Dayhoff and Schwatz (1981) using the $F_O$ (proteolipid) subunit of mitochondrial, chloroplast and *E. coli* ATPases. This portion of the tree also reflects the endosymbiotic origins of mitochondria and chloroplast from prokaryotes.

The top right corner of Fig. 5.6 shows the 57 kDa polypeptides of *Arabidopsis* and *Neurospora* vacuolar membrane ATPases branching out from the same node. This concurs with the extensive biochemical (Poole *et al.* 1984, Bowman and Bowman, 1982) and sequence (Table 5.1) similarities found between the two endomembrane $H^+$-ATPases. Structural, functional (see Chapter 1) and immunological studies (see Chapter 4) suggest that the common point of origin will extend to $H^+$-ATPases of yeast vacuolar membranes, chromaffin granules, clathrin-coated vesicles and lysosomes.

The $\beta$ subunit of *Sulfolobus* is placed between the nodes leading to either the $F_OF_1$ or vacuolar types of ATPases. The structure and function of the archaebacterial ATPases (see Chapter 1) also suggest that they have an intermediate relationship between the two other classes of ATPases. Phylogenetic analysis of ribosomal RNA indicates that eubacteria and archaebacteria have entirely separate lineages evolving from a common ancestor (Woese and Fox, 1977). This suggests that the root of the tree should be the ATPase of the hypothetical universal ancestor (progenote) and not the archaebacterial ATPase. However, considering the apparent antiquity of archaebacteria (Woese and Fox, 1977), perhaps

their ATPases reflect more closely the as yet unidentified prototype ATPase. This is reflected in Fig. 5.6 by placing *Sulfolobus* to the far left.

# 6.0 General Discussion

This thesis describes the isolation and characterization of a higher plant vacuolar membrane $H^+$-ATPase, composed of at least three polypeptides.

A 16 kDa polypeptide was identified by DCCD labeling and by co-purification with ATPase activity (Fig 3.4). The subunit's size and its interaction with DCCD suggested (by analogy with the $F_OF_1$ ATPases) that it forms part of a membrane-spanning proton channel. Chloroform/methanol extraction has confirmed the 16 kDa polypeptide as an integral membrane protein (Rea *et al.*, 1987; Kaestner *et al.*, 1987). In the case of the $H^+$-ATPase of clathrin-coated vesicles, participation of the 16 kDa subunit in a transmembrane proton channel has been verified by DCCD-sensitive facilitation of proton diffusion in a reconstituted vesicle system (Sun *et al.*, 1987). The 16 kDa subunit and subunit C of the $F_OF_1$ ATPases are similar in their ability to bind DCCD, their solubility in chloroform/methanol, and their function as transmembrane proton channels. However, they differ in their sensitivity to DCCD (see Chapter 1) and in their size (16 vs 8 kDa). While 12 copies of subunit C are required per $F_OF_1$ ATPase (Schneider and Altendarf, 1987) an average of 6 copies of the 16 kDa has been calculated for vacuolar ATPases (Kaestner *et al.*, 1987; Michael Forgac, personal communication). A correlation between the

doubling in size and the requirement of half the number of subunits suggests that the 16 kDa subunit may have arisen through gene duplication. These ideas are supported by the recent cDNA sequencing of the 16 kDa polypeptide from chromaffin granules (Nathan Nelson, personal communication)

Photoaffinity labeling with BzATP identified a 57 kDa polypeptide as a nucleotide-binding subunit of the vacuolar $H^+$-ATPase (Manolson *et al.*, 1985). It has been suggested that this result contradicts the reports which used azido-ATP to identify the larger (67 kDa) polypeptide as containing the catalytic site (Moriyama and Nelson, 1987a; Uchida *et al.*, 1988). These results do not contradict but rather complement each other. BzATP, although a potent inhibitor of hydrolysis, is itself not hydrolyzed unless present in excess (greater than 50 mM) while azido-ATP is hydrolyzed with a $K_m$ of 1.5 mM (Manolson, unpublished data). The enzyme kinetics using BzATP as an inhibitor displayed positive cooperative kinetics with respect to ATP concentration (Fig. 3.1) suggesting that BzATP is interacting with a regulatory ATP-binding site distinct from the catalytic site. While no one has done the same type of kinetics using azido-ATP, kinetic analysis of NBD-Cl labeling has identified the larger (67 kDa) polypeptide in corn as the catalytic subunit (Randall and Sze, 1987). Perhaps the differences in enzyme kinetics and subunit labeling can be explained by the steric differences between the two photoaffinity probes. The benzophenone group of BzATP is substituted at the 3'-hydroxyl position of the ribose moiety while the azido group of azido-ATP is attached to carbon 8 of

the purine moiety. Randall and Sze (1987) observed ATP-protectable, [$^{14}$C]-NEM binding to the lower (57 kDa) subunit, confirming the presence of a nucleotide binding site.

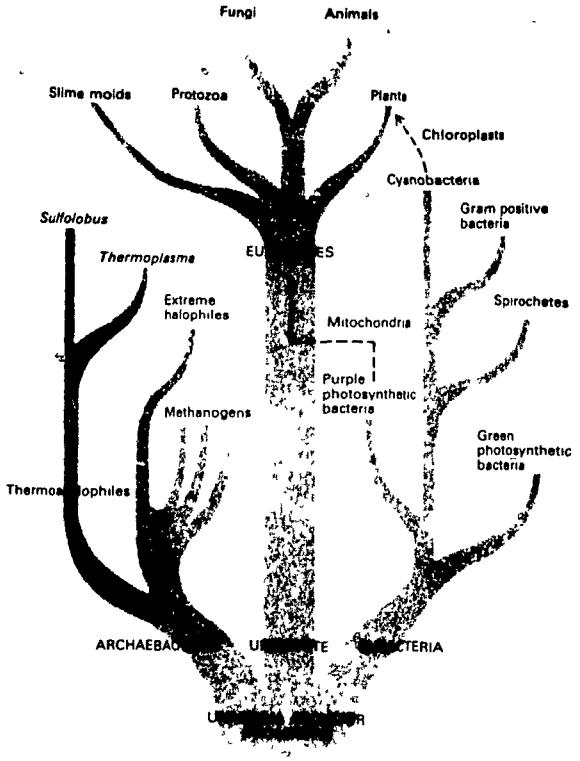
The 57 kDa polypeptide of endomembrane ATPases and the $\alpha$ subunit of the $F_0F_1$ ATPases appear to be functionally homologous in that both subunits contain nucleotide binding sites with supposed regulatory functions. Anti-57 antibodies binding to the $\alpha$ subunit of *E. coli* and to the "a" subunit of *Clostridium pasteurianum* (Fig 4.2) support this correspondence between subunits. Although neither the $\alpha$ nor the 57 kDa subunit contains the catalytic site for its respective ATPase, these subunits may still be involved in hydrolysis. PAF-stimulated phosphorylation of the lower (57 kDa) subunit has been shown to promote nitrate sensitive ATPase activity in zucchini microsomes (unpublished work in collaboration with Georg Martiny-Baron). In the case of the bovine $F_0F_1$ ATPase, photoaffinity labeling with BzATP has led to speculation that the catalytic site is formed between or at the interface of the $\alpha$ and $\beta$ subunits (Williams and Coleman, 1981).

Initial characterization of ATPases from various endomembrane organelles revealed differences in physiological properties (section 1.4) and in the number and molecular weights of the subunits (Table 1.3). Antibodies raised against the two nucleotide-binding subunits in red beet vacuolar $H^+$-ATPase (57 and 67 kDa) were shown to cross-react with the corresponding subunits of the $H^+$-ATPases from bovine chromaffin granules, bovine clathrin-coated vesicles, yeast vacuolar membranes (Fig. 4.2) and rat liver lysosomal membranes (data

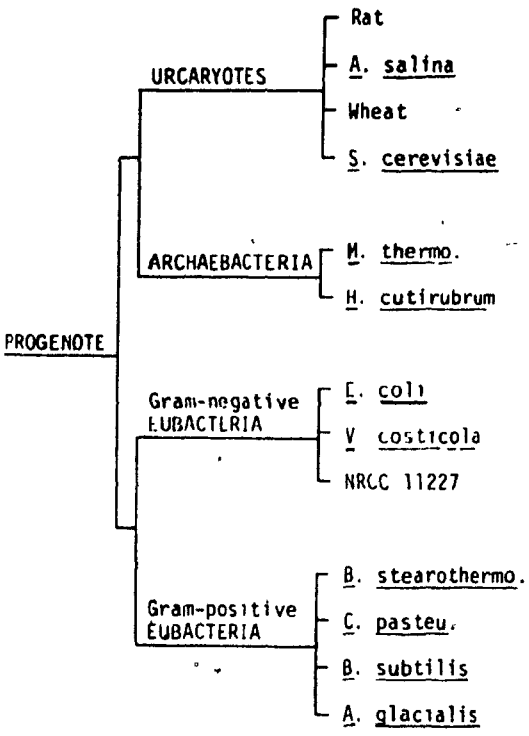
not shown). This was the first demonstration of the structural conservation amongst endomembrane $H^+$-ATPases from different organelles and different phyla confirming the hypothesis of a common class. The anti-57 sera also reacted with nucleotide-binding subunits from the ATPases of *Clostridium pasteurianum*, and *E. coli* suggesting a relationship between the endomembrane and $F_OF_1$ ATPases. Primary sequence comparisons of ATPase subunits left no doubt as to the common origin of endomembrane ATPases and to their link with archaebacterial and $F_OF_1$ ATPases (Chapter 4). No other ATP-utilizing enzyme showed any significant homology to the ATPase subunits (Table 5.1). This suggests that the sequence similarities amongst the ATPases reflect more than common function but indicate a common evolutionary origin. Phylogenetic analysis of sequence homologies show the endomembrane ATPases as having a closer evolutionary relationship to the archaebacterial ATPases than to the $F_OF_1$ ATPases (Fig. 5.6).

Without placing too much emphasis on mere speculation, one can attempt to extrapolate the phylogenetic data of the ATPases to the organisms and organelles in which they reside. First, it is necessary to fit the data of the ATPases to the known phylogeny of their respective organisms. This is no easy task since the phylogenetic relationship of archaebacteria, eubacteria, and eukaryotes has become a highly debated issue. The established view (Woese and Fox, 1977) proposes that archaebacteria, eubacteria, and eukaryotes form three evolutionarily equidistant aboriginal lines of descent stemming from one common ancestor, the progenote (Fig. 6.1, A). The

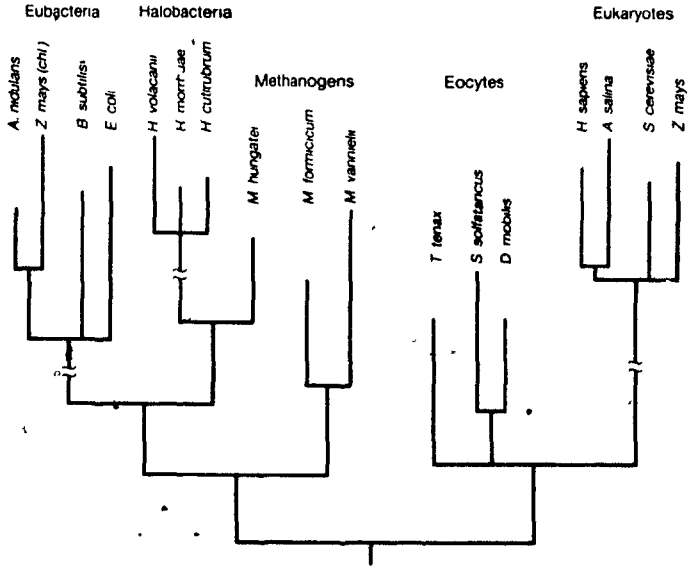
Figure 6.1 **Phylogenetic trees relating the primary kingdoms.** Panel A constructed by Woese (1981), panel B constructed by Yuguchi *et al.*, (1980) and panel C constructed by Lake (1988).


Fungi
Animals
Slime molds
Protozoa
Plants
Chloroplasts
Cyanobacteria
Gram positive bacteria
Sulfolobus
Thermoplasma
Extreme halophiles
Mitochondria
Spirochetes
Purple photosynthetic bacteria
Methanogens
Green photosynthetic bacteria
Thermo ophiles
ARCHAEBA
UR TE
TERIA

A


Rat
URCARYOTES
A. salina
Wheat
S. cerevisiae
ARCHAEBACTERIA
M. thermo.
H. cutirubrum
PROGENOTE
Gram-negative EUBACTERIA
E. coli
V. costicola
NRCC 11227
Gram-positive EUBACTERIA
B. stearothermo.
C. pasteu.
B. subtilis
A. glacialis

B


Eubacteria
Halobacteria
Methanogens
Eocytes
Eukaryotes
A. nidulans
Z. mays (chl.)
B. subtilis
E. coli
H. volcanii
H. morrhuae
H. cutirubrum
M. hungatei
M. formicicum
M. vannielii
T. tenax
S. solfataricus
D. mobilis
H. sapiens
A. salina
S. cerevisiae
Z. mays

C

common origin of the ATPases could then be explained if the progenote contained the prototype ATPase. However, this interpretation does not agree with the phylogenetic data in Chapter 5. Woese's evolutionary scheme would predict the three groups of ATPases (endomembrane, archaebacterial and $F_0F_1$) to be equally dissimilar from each other. The data in Fig. 5.6 show the endomembrane and *Sulfolobus* ATPases as being more similar to each other than to the $F_0F_1$ ATPases. This discrepancy could possibly be accounted for by the changing function of the ATPases. Although the role of *Sulfolobus* ATPase has yet to be directly established, indirect evidence suggests that it acts as an ATP synthase (Konishi *et al.*, 1987). The *Sulfolobus* ATPase would then be expected to show more similarities to the $F_0F_1$ ATP synthetases than to the proton pumping endomembrane ATPases. This again does not agree with the data in Chapter 5. A second possibility to fit Woese's evolutionary scheme is to suggest that the similarities between the endomembrane and *Sulfolobus* ATPases reflect formation of the various endomembrane organelles through endosymbiosis of archaebacteria. An endosymbiotic formation of endomembrane organelles has been proposed by Al-Awqati (1986). Although the endosymbiotic origin of mitochondria and chloroplast from eubacteria explains the extensive homologies amongst $F_0F_1$ ATPases (Schwartz and Dayhoff, 1978), this seems an unlikely explanation for the endomembrane organelles. The evidence, apart from the $F_0F_1$ ATPases, for the endosymbiotic formation of mitochondria and chloroplasts is extensive, including the retention of prokaryote-like genes as well as

sequence homologies of rRNA, tRNA, ferredoxins, and cytochromes (Dayhoff and Schwartz, 1981; Schwartz and Dayhoff, 1981). No other evidence, apart from ATPase subunit similarities, has so far been found between endomembrane organelles and archaebacteria or eubacteria. While mitochondria and chloroplasts are self-contained, endomembrane organelles are tightly interwoven within the eukaryotic cell by endocytic pathways (Mellman, 1986). That the endomembrane type of ATPase is found in such a wide variety of organelles argues against the idea of this class of ATPases being endosymbiotic late-comers to the evolutionary life of eukaryotes.

Woese's three equidistant primary kingdoms theory has been challenged. Yaguchi *et al.* (1980) found partial amino acid sequences of the archaebacterial ribosomal A protein to be far more similar to the eukaryotic type than the eubacterial type. This led Yaguchi to propose that the progenote separates initially into two lines of descent, the eubacteria and the common ancestor of archaebacteria and eukaryotes (Fig. 6.1, B). The recent cloning and complete sequencing of the ribosomal A protein gene from the archaebacterium *Halobacterium halobium* has confirmed Yaguchi's initial observation (Itoh *et al.*, 1988). The algorithm with which Woese compared the 16S and 18S rRNA sequences has recently been criticized as being biased by unequal rate effects (Lake, 1988). Lake reanalyzed the rRNA data using an evolutionary parsimony algorithm and proposed that the progenote divides initially into not three, but two branches. One branch leads to the eubacteria, halobacteria, and methanogens, and the other branch leads to the eocytes and

eukaryotes (Fig. 6.1, C). The eocytes are defined as a group of extremely thermophilic, sulphur-metabolizing, anucleate cells (Lake, 1984). Yaguchi and Lake differ as to the placement of the halobacteria and methanogens, but they both have thermoacidophiles and eukaryotes branching off together from the progenote. If one now considers the progenote as containing the prototype ATPase, either Yaguchi's or Lake's evolutionary scheme would predict the close relationship between the endomembrane and *Sulfolobus* ATPases. Thus, the sequence homologies of the ATPase subunits concur with the thesis of either Yaguchi or Lake that the early ancestor of eukaryotes was an archaebacterial type of organism. Perhaps endomembrane organelles arose through endocytosis of the plasma membrane. Endocytosis would result in the archaebacterial plasma membrane-bound $H^+$-ATPase being found on a cytoplasmic membrane-bound vesicle, with the active site of the ATPase in the cytoplasm and protons being pumped into the lumen of the vesicle, as is the case for the endomembrane ATPases. It is not yet known whether the P-type ATPase characteristic of eukaryotic plasma membranes also occurs in the eocytes, although it is found in the eubacteria (Hesse *et al.*, 1984 and Furst and Solioz, 1986), and there is some evidence for its presence in methanogens (J. Konisky, personal communication). Thus both the P-type and the $F_OF_1$ or endomembrane-type may have been present on the plasma membrane of the progenote, becoming largely (though not completely) segregated during the evolution of the endomembrane system.

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## Contributions to Original Knowledge

--I was one of the first investigators to demonstrate the multimeric structure of an endomembrane ATPase. This work was first presented in Manolson, M. F., Poole, R. J., (1984), MOLECULAR STRUCTURE OF THE TONOPLAST ATPASE OF *BETA VULGARIS* L., Proc. 26th Annual Meeting of Can. Soc. Plant Physiol., Vancouver, p.15. The first refereed publication on this topic was by Uchida *et al.*, in 1985.

--I was the first investigator to publish any nucleotide-binding data for endomembrane ATPases and I am still the only investigator to have published biochemical data demonstrating a nucleotide-binding site on the "57 kDa" polypeptide of endomembrane ATPases.

--I was the first investigator to demonstrate common structural features for endomembrane $H^+$-ATPases of different organelles and to show that a possible link exists between the endomembrane and $F_0F_1$ ATPases. This was first presented in Manolson, M. F., Poole, R. J., IMMUNOLOGICAL COMPARISON OF ENDOMEMBRANE $H^+$-ATPASES FROM HIGHER PLANTS AND ANIMALS, Membrane Protein Symposium, San Diego, (1986)

--I was one of the first investigators to sequence one of the polypeptides from an endomembrane ATPase and to show sequence homologies between endomembrane, archaebacterial, and $F_0F_1$ ATPases. This was presented at the International Workshop on Membrane ATPases, Osnabruck, Germany in February, 1988, concurrently with the first presentation of sequence data from Lincoln Taiz, Emma Jean and Barry Bowman, and Masasuke Yoshida.