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STRUCTURE, FUNCTION AND REGULATION OF THE H-ATPase IN PLANT TONOPLAST MEMBRANE

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Title

Structure, function and regulation of H⁺-ATPase in plant tonoplast membrane

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C. Abstract

The kinetic properties of the membrane-bound and of the partially purified tonoplast proton ATPase were evaluated with respect to chloride, Mg^{2+} and Mn^{2+} ions. Metal-ATP complex was found to be the true substrate while free cations inhibited the activity in the two forms of the enzyme. The inhibition generated a change in both the V_{max} and the K_m and was induced by interaction through two cationic binding sites. Chloride ions almost doubled the V_{max} but did not change the K_m . Five to ten fold decrease in the K_i for inhibition by free Mg^{2+} and Mn^{2+} ions and increase in the K_i for the inhibition by nitrate were induced by chloride ions. Chloride ions affected nitrate inhibition by interacting directly with the same anionic binding site. Chloride indirectly modulated the binding of the substrate and the free cations.

The mechanism of sulfite stimulation was also investigated. Nonlinear hydrolysis kinetics were observed, and sulfite-stimulation was limited to the steady state phase of the reaction. At $MgATP$ concentrations < 1 mM, the reaction was linear and sulfite inhibited activity due to a 5-fold increase in the K_m for substrate. The stimulation of hydrolysis was due to slip, since no stimulation of proton transport was observed.

Control of V-ATPase activity by oxidation-reduction of disulfide bridge in tonoplast vesicles was investigated with the aid of specific oxidizing reagents. About 70% of the activity was inhibited by treatment with oxidized glutathione. The inhibition was reversed by reduction with DTT. Identification of the essential cysteines which are involved in the regulation of the catalysis by formation of disulfide bond and their subunit location was done by N-ethylmaleimide (NEM) modification. NEM is specific cysteine modifier which inhibits the catalytic activity. [^{14}C]NEM labeled subunit A and the labeling was greatly reduced by pre oxidation of the enzyme with oxidized glutathione.

The role of highly conserved cysteine residues in the catalytic site was investigated by constructing a series of Cys $\rightarrow$ Ser mutants in A subunit of yeast V-ATPase. Changing Cys261 to serine did not affect catalytic activity but completely eliminated inhibition by NEM. Thus Cys261, which is located next to the nucleotide-binding motif "GKT", is the site of NEM binding. Changing either Cys284 or Cys538 to serine resulted in a loss of activity, demonstrating that they are essential.

D. Objectives.

Kinetic analysis was intensively used by the two investigators for the study of the mechanism of enzyme action, as originally proposed. The results of these studies gave a new insight into the mode of action of the enzyme. Regulation by cations, ions, substrate and product was another major objective that was successfully pursued by the two collaborators. The study of the effects of anions such as chloride, sulfite and nitrate and the study of the interaction between anions and divalent cations resulted in a new and better understanding of the mode of regulation.

Although we had great and unexpected difficulties in purification of the enzyme, as we reported in the last two progress reports, we finally obtained purified protein. Apparently, as is reported by other groups which attempt purification, the major problems are due to the difficulty in obtaining a source which can give large amount of starting material and in getting high yield of the purified enzyme. The lack of adequate quantities of purified enzyme prevented real progress in the attempts to reconstitute the enzyme and to raise antibodies against individual subunits. The antibodies against the A subunits which were finally raised are currently in use. These were two of the approaches originally proposed for the study of the role of individual subunits in catalysis.

To overcome these problems, as we proposed in our plans for the third year, the objectives of the proposal were modified to take advantage of exciting new results and ideas emerging from our mutual sabbaticals in the laboratory of Nathan Nelson at the Roche Institute of Molecular Biology. During this time a molecular analysis of the cysteine residues of the catalytic subunit of the yeast vacuolar ATPase was undertaken by L.T. This led to a discussion of the role of oxidation/reduction in regulation. Accordingly, we have focused our work on the analysis of the cysteine residues and the mechanism of regulation by oxidation-reduction.

E. Scientific Report

Introduction

Modulation of ATPase Activity by Divalent Cations and Anions in The Membrane Bound and The Soluble Tonoplast H⁺-ATPase from Mung Beans The tonoplast H⁺-ATPase is an electrogenic pump which generates an electrochemical potential of protons across the tonoplast membrane and maintains a low pH inside plant vacuoles (1). This potential is utilized for the energization of secondary solute transport into the vacuole. The enzyme is constructed of several subunits, some of which are integral membrane proteins and the others are attached to the membrane. As in the case of the mammalian and the yeast vacuolar ATPases (2), 7 to 10 subunits were suggested to compose the enzyme in plants (3,4). Similar to other V-ATPases the plant pump is stimulated by some anions with Cl⁻ being the most effective. Other anions, most specifically NO₃⁻ inhibit the activity. Vanadate or azide which are potent inhibitors of the P-ATPases and the F-ATPases, respectively (1), are poor inhibitors of this enzyme. V-ATPases

were shown to require divalent cations and anions for enzymatic activity (1,2). Magnesium ions are activators of the enzyme and are required for a formation of MgATP complex, which serves as the true substrate. Chloride ions stimulate pumping activity by direct interaction with the enzyme and by functioning as activators of the hydrolytic activity. They also serve as the major symport anion which is required for the formation of a pH gradient by this electrogenic proton pump. The transport of Cl^- ions to the lumen of the vesicles is probably mediated through a chloride channel which is separated from the ATPase (1,2). The interaction of cations and anions is of special physiological significance in tonoplast ATPase since this pump is involved in regulation of plant cell turgor and in adaptation to salinity.

Detailed studies with tonoplast vesicles indicated that Cl^- ions stimulated ATPase activity by increasing the V_{max} without altering the K_{m} for MgATP (5). Yet an inhibition by free Mg^{2+} ions was also induced by the stimulating anion. Inclusion of protonophore seemed to change the response to Cl^- ions, inducing a decrease in the K_{m} of ATP instead of changing the V_{max} (6). The change in V_{max} in the absence of protonophore could be only partially due to the dissipation of the membrane potential by the Cl^- ions. The membrane potential generated by proton pumping was shown to inhibit ATPase activity (7). It seems that the mode of action of Cl^- ions was not completely resolved. Other factors such as the anionic effect on the inhibition by divalent cations had to be considered. There are also conflicting reports on the effect of Cl^- on NO_3^- inhibition. Chloride ions were reported to increase the inhibition by NO_3^- (6,8), to have a slight effect (7) or to decrease the inhibition (9). Although the interaction between the two anions could be a result of binding to a common anion binding site, the stimulation of the pH gradient by Cl^- was shown to be noncompetitively inhibited by NO_3^- ions (7). The understanding of the nature of the interaction of cations, anions, substrate and products with the enzyme can be used as a powerful tool in the investigation of the mode of action and regulation of the enzymatic activity. In this work ATPase activity was determined in tonoplast vesicles and in the soluble, partially purified enzyme. The idea was to distinguish between the primary interactions, due to the direct effect on catalysis, from the secondary effects which might be a result of the membrane potential formed by proton pumping. The conflicting reports regarding the mode of action of Cl^- and the potential of such studies required an expansion of the current knowledge about the effect of Cl^- ions on the activation and the inhibition induced by Mg^{2+} and Mn^{2+} ions. These two cations were shown to equally activate the enzyme by forming divalent metal ATP complexes but differed in their capacity for inhibition as free cations. The modulation of the activity was due to the interaction of divalent cations with multiple binding sites on the enzyme. The interaction between the stimulation caused by Cl^- and the inhibition by NO_3^- indicated that the two anions might have a common binding site.

Nonlinear Kinetics and Sulfite Regulation of the Vacuolar H^+ ATPase. F-type ATPases have been shown to exhibit nonlinear hydrolysis kinetics in which an initial rapid rate is followed by a

slower steady state rate (10,11). The decline in the initial rate has been shown to be caused by a turnover-dependent inactivation brought about by the tight-binding of ADP at the catalytic site. Stimulatory oxyanions such as sulfite, bicarbonate and phosphate have been shown to exert their effects through a re-activation of the inactive complex, i.e. the oxyanion promotes the release of tightly-bound nucleotide. Kinetic studies by Boyer and colleagues have shown that sulfite increases V_{max} without affecting K_m (12). This is consistent with the above model in which sulfite reactivates inactive enzyme. Recently, Schobert (13) demonstrated that the H^+ -ATPase of *Halobacterium* also exhibits nonlinear kinetics and is stimulated by sulfite. Sulfite stimulation occurs during the steady state phase of the reaction and appears to be due to a reactivation of inactive enzyme, similar to the situation in the F-ATPases. Since eukaryotic V-ATPases are closely related to the archaeobacterial-type H^+ -ATPase, and distantly related to the F-ATPases, one might expect V-ATPases to show similar nonlinear kinetics and sulfite stimulation. On the other hand, V-ATPases function as ATP-driven proton pumps while F-type and archaeobacterial-type ATPases function as ATP synthases, so their regulation might be quite different.

Sulfite-Activation and $\Delta \mu H^+$. Although sulfite is known to stimulate ATP hydrolytic activity in the F-ATPases, there is little information concerning its effects on proton transport. Studies with the mitochondrial and chloroplast ATPases have shown, however, that the formation of the inactive form of the enzyme is promoted by the collapse of $\Delta \mu H^+$, and is reversed by the reimposition of $\Delta \mu H^+$ (14). This makes sense for an enzyme whose function is to synthesize ATP. In the absence of $\Delta \mu H^+$ to drive ATP synthesis, the enzyme could operate in reverse. Inactivation prevents wasteful hydrolysis of ATP when the membrane is not energized. The function of the vacuolar ATPase is to generate $\Delta \mu H^+$. In this case the enzyme should be active when $\Delta \mu H^+$ is low and shut off when $\Delta \mu H^+$ exceeds a certain amount. To better understand the regulatory role of $\Delta \mu H^+$ we investigated the effect of $\Delta \mu H^+$ on sulfite activation and the effect of sulfite on proton transport.

The Role of Cysteines in the Catalytic Subunit of the Yeast V-ATPase

We have been using site-directed mutagenesis to study the role of cysteine residues of the A subunit of the yeast vacuolar ATPase. As shown in Figs. 19 A and B, Cys 261, Cys 284 and Cys 538 are highly conserved among the eukaryotic V-ATPases and, in some archaeobacterial-type ATPases as well. However, they are not present in the beta subunits of the F-type ATPases (15). In eukaryotes, Cys 261 (or the equivalent residue) is located adjacent to the proposed phosphoanhydride-binding motif, "GKTV", which is widely occurring in the superfamily of nucleotide-binding proteins. The equivalent residue is a serine in archaeobacteria, and valine in eubacteria (16). Cys 284 is located downstream from the "GKTV" motif ("GERGNE") and is proximal to a region that has been shown to bind DCCD in F-ATPases. It is the only cysteine that is universally conserved in all eukaryotes and all archaeobacteria. The equivalent residue is a valine in the eubacteria. Cys 538 is located near the carboxy terminus. The equivalent residue is

conserved in all eukaryotes and in some archaebacteria, but not all. The first question we wished to address was whether or not these cysteines are required for catalytic activity. To determine this, the cysteines were altered to serine and the mutant cells were tested for their ability to grow at pH 7.5 vs. pH 5.5. Only cells with an active V-ATPase can grow at pH 7.5. A nonconserved cysteine (Cys 254) was also tested as a control.

Control of V-ATPase activity by oxidation-reduction of disulfide bridge in tonoplast vesicles. V-ATPases from various organisms is inhibited by NEM (1,2), which is specific cysteine modifier. In V-ATPase from beef chromaffin granules and from clathrin-coated vesicles the extent of inhibition by NEM was reduced when the enzyme was pre oxidized (2). It seemed that oxidation-reduction of disulfide bridge could be important means for regulation of the enzyme. We therefore investigated this phenomenon with the aid of specific cysteine oxidizing reagents.

Experimental procedure

Isolation of tonoplast vesicles. Tonoplast vesicles were prepared from mung beans (*Vigna radiata* L.) as described earlier (17). Essentially 250 g hypocotyls of etiolated seedlings were excised and homogenized for 1 min in Waring blender in a medium containing 75 mM tris-Cl (pH 7.6), 0.25 M sorbitol, 4 mM EGTA, 2 mM DTT, 0.2 % BSA and 1mM phenylmethylsulfonyl fluoride at 4°. The homogenate was filtered through cloth and centrifuged at 1500 x g for 30 min. The supernatant was centrifuged at 10,000 x g for 30 min. The supernatant was collected and centrifuged at 80,000 x g for 30 min. The pellet was suspended in 5.5 ml of a medium containing 10 mM tris-MES (pH 7.3) 0.25 M sorbitol, 1 mM EGTA and 1mM DTT. The suspension was layered on top of a 5.5 ml of the same solution containing 6 % dextran-T 70 and was centrifuged at 105,000 x g for 110 min in a Beckman SW 40 rotor. The turbid interface was collected, diluted 5 folds in the suspension medium and centrifuged at 140,000 x g for 30 min. The pellet was resuspended in 3 ml of the same medium and stored at -70°.

Partial purification of tonoplast ATPase. Tonoplast vesicles were washed with 5% triton X-100 as described in Ref. 4 and the pellet solubilized in a medium containing 20 mM MOPS-KOH (pH7.5), 1 mM EGTA, 1mM DTT, 2 mM MgCl₂, 20% glycerol and 2mg/ml lysophosphatidylcholine. The dissolved enzyme was separated from the membrane by centrifugation, resuspended in the same medium lacking the detergent and stored at -70°.

Assay of ATPase activity. Activity was assayed in a medium containing 30 mM tris-MES (pH 7.5), 50 mM KCl, 3 mM MgSO₄, 3 mM ATP, 10 to 20 µg protein in a total volume of 0.5 ml. Dissociation constants (18) were used for the calculations of the concentrations of MgATP and MnATP complexes. When activity was measured in tonoplast vesicles 0.5 mM NaN₃ was added. After 30 min at 30° the reaction was terminated by addition of a reagent used for spectrophotometric assay of the released Pi (19). Protein concentration was determined according to the method as described in Ref. 20.

Data analysis: The kinetic parameters of ATPase activity were determined using Eq. 1 and 2 as

described in Ref.21. The data were fitted to the equations by the "KaleidaGraph 2.1" a graphic software system by Synergy Software, Reading, USA. Eq.1 is a Michaelis-Menten expression of a linear mixed type inhibition as presented in Eq.3, and Eq. 2 is the expression for uncompetitive inhibition as presented in Eq.4.

$$\text{Eq. 1} \quad \frac{v}{V_{\max}} = \frac{[S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S] \left(1 + \frac{[I]}{\alpha K_i}\right)}$$

$$\text{Eq. 2} \quad \frac{v}{V_{\max}} = \frac{[S]}{K_m + [S] \left(1 + \frac{[I]}{K_i}\right)}$$

Where S, I and E are substrate, inhibitor and enzyme, respectively; v and $V_{\max}$ are velocity and maximal velocity, respectively; K_s , K_i and K_m are dissociation constants of the substrate, the inhibitor and Michaelis constant, respectively. α is the factor by which K_s is changed when I occupies the enzyme.

Preparation of yeast vacuoles, solubilization of V-ATPase and assay of proton pumping and ATPase activity. Vacuoles were prepared as previously described in Ref. 22. For solubilization, five milligrams of vacuolar membranes were washed three times with EDTA buffer (10 mM TRIS-HCl pH 7.5, 1 mM EDTA, 2 mM DTT) and resuspended in solubilization buffer (10 mM Tris/Mes pH 7.5, 1 mM EDTA, 2 mM DTT, 0.5 mM PMSF, 10% glycerol). The membranes were solubilized with Zwittergent 3-14 in solubilization buffer and loaded onto a ten ml linear 20-42% glycerol gradient prepared in solubilization buffer supplemented with 0.005% Zwittergent 3-14. The gradient was centrifuged overnight and 0.6 ml fractions were collected as described by Moriyama and Nelson (23). ATPase and proton pumping experiments were performed as previously described by us (24, 25).

Oxidation by glutathione and labeling of tonoplast ATPase with [^{14}C] NEM. Samples of tonoplast vesicles or of partially purified enzyme containing 15 μg protein were passed through G-50 centrifuged columns equilibrated with 20 mM tris-MOPS (pH 7) and 1 mM EGTA. Glutathione, in a final concentration of 10 mM was added for a 2 h incubation at 25 $^{\circ}$ in a total volume of 20 μl . NEM, 1 μl from 2M stock solution was added (0.1 mM, final concentration) for 10 min incubation at 25 $^{\circ}$. The same procedure was used for samples which were treated with NEM without pre incubation with glutathione. The reaction was terminated by addition of 1 μl DTT to a final concentration of 10 mM. For assay of activity reaction medium was added to a total volume of 0.5 ml as described above. For radioactive labeling [^{14}C] NEM (2 $\mu\text{Ci}/\mu\text{moles}$) was used.

SDS gel electrophoresis on a 10% polyacrylamide gel was done as described by Laemmli (25a). Following separation the gel was fixed in 25% methanol and 7% acetic acid for 20 min, and soaked in 1 M sodium salicylate for 30 min for amplification of radioactivity. The gel was dried under vacuum and exposed to Kodac XAR 5 film for 1 week at -70 $^{\circ}$.

Analysis of site directed mutations in cysteines of ATPase A subunit from yeast vacuols. Mutational analyses were carried out according to the methods of Nelson and colleagues using the haploid yeast strain (W303-1B) (26). The strain contained a disrupted copy of the *vma1* gene. Altered copies of the gene were introduced via the yeast shuttle vector "YPNTR" (26). Transformants were selected on tryptophan-minus minimal medium plates and tested on pH 7.5 and 5.5 YPD-agar plates. Yeast cells with an active V-ATPase can grow at both pH's, whereas mutant cells with inactive V-ATPases can grow only at pH 5.5 (permissive pH).

Results

The dependence of ATPase activity on Mg^{2+} and Mn^{2+} ions. The kinetic studies were done with tonoplast vesicles prepared from mung bean hypocotyls. These vesicles had high ATPase activity which was mostly nitrate and NEM sensitive. Small fraction of the total hydrolytic activity was probably due to mitochondrial contaminants since it could be inhibited by azide. Therefore azide was regularly included in the assays to eliminate mitochondrial contribution to the measured activity. Kinetic analysis of substrates and activators is a potent approach to identification of binding and catalytic sites involved in catalysis and regulation. Initially the dependence of the rate of the tonoplast ATPase activity on divalent metal ions was shown to describe an optimal response in the presence of chloride ions. The activities for both Mg^{2+} and Mn^{2+} ions peaked at concentrations which were close to the concentration of ATP and declined at higher concentrations with a much stronger inhibitory effect exerted by manganese than by magnesium ions (Fig. 1).

Inhibition by free Mn^{2+} ions. Although Mn^{2+} ions are analogs of the physiologically functioning Mg^{2+} ions (5) the former ions were initially utilized as probes for the

catalytic properties since they showed stronger inhibitory effect. Such an effect could be helpful in the analysis of metal and nucleotide interactions with the enzyme. Evaluation of the data from the optimum curve indicated that the true substrate was probably a metal-ATP complex and that the free metal had an inhibitory effect. In order to verify these indications ATPase activity was measured as a function of the concentration of MnATP complex in the presence of various concentrations of the free metal. As was shown earlier for Mg^{2+} ions (5), the metal nucleotide complex was indeed the true substrate, as saturation response curves were obtained when the velocity was measured as a function of MnATP as substrate (Fig. 2). The inhibition by the free metal was not overcome at high substrate concentrations. A double reciprocal plot of the activity versus substrate concentration indicated that the slope of the plot did not change but the $1/v$ intercept increased as a function of the increase in the concentration of the free Mn^{2+} ions (Fig. 3). Two of the possible kinetic interpretations of such behavior could be either an uncompetitive or a hyperbolic mixed type inhibition (21). The two can be distinguished by analysis of the effect of inhibitor concentration of the $1/v$ intercept. A replot of the $1/v$ intercept versus the concentration of free Mn^{2+} described a linear function (Fig. 3, inset) which is indicative for uncompetitive inhibition. Support for the suggestion that free divalent metal ions interact with the enzyme in a manner which modulates both K_m and V_{max} came also from the finding that at low inhibitor concentrations a linear mixed type inhibition was observed (Fig. 4). Changes in both K_m and V_{max} are characteristic of this mode of inhibition as in the case of uncompetitive inhibition. Switching from one mode of inhibition to another might be caused by binding of divalent metal ions to two inhibitory sites. The first is an high affinity site which effects a linear mixed type mode. When the first site is saturated, filling of the second site induces uncompetitive inhibition.

Modulation of the kinetic properties of ATPase activity by divalent metal cations. In uncompetitive and mixed type inhibition there is a change in the V_{max} and the K_m because of the binding of both the free metal ion and the substrate to the enzyme thus producing the inhibitory complex. Therefore according to Eqs. 1 and 2 a fit of $1/v$ versus $1/S$ at various inhibitor concentrations can be used only for calculations of apparent rate and equilibrium constant. Secondary plot of $1/V_{max}$ apparent and $1/K_m$ apparent versus free inhibitor concentrations can be fitted to the equations to give the true kinetic properties of the enzyme. From a fit of $1/V_{max}$ apparent of the activity of ATPase in tonoplast vesicles at various concentrations of free Mn^{2+} ions (Fig.3, inset) the V_{max} and the K_i of uncompetitive inhibition by the free ion were calculated (Table I). From the fit of K_m apparent at various free metal concentrations the K_m was calculated. Similar calculations were used to obtain the K_m , V_{max} , K_i and the α factor for the mixed type inhibition (Table I). The K_i value of 3.9 ± 0.02 mM for mixed type inhibition was slightly lower than the K_i of 5.2 ± 0.3 mM of uncompetitive inhibition by free Mn^{2+} ions. The

lower K_i could be a result of binding to a high affinity binding site which induces mixed type inhibition. However similar K_m and V_{max} values were obtained for regardless of the type of inhibition.

Inhibition of soluble partially purified enzyme by Mn^{2+} ions. ATPase activity measured in tonoplast vesicles, under the described experimental conditions, was shown to be primarily contributed by the V-ATPase. Yet it was essential to confirm the results by assay of the partially purified enzyme before mechanistic conclusions could be drawn. In these studies only partially purified enzyme was used because the yield of highly purified protein was very low and the quantities were insufficient for kinetic studies. The major contaminant in the preparation was pyrophosphatase as seen in the SDS gel electrophoresis and from activity assay. Since ATP is not hydrolyzed by pyrophosphates (27) its presence did not interfere with the analysis of the ATPase activity. ATP hydrolysis was almost completely inhibited by nitrate and was NEM sensitive. These inhibitors are characteristic of the vacuolar ATPase. Azide and molybdate which inhibit mitochondrial ATPase and phosphatase, respectively, did not inhibit the activity.

In the partially purified ATPase, as in the membrane bound enzyme, MnATP proved to be the true substrate and free Mn^{2+} ions inhibited the activity. Mixed type and uncompetitive inhibitions were induced by low and high free Mn^{2+} , respectively. The differences in affinity were also expressed in a K_i of 0.9 ± 0.02 mM for uncompetitive inhibition which was slightly higher than the K_i of 0.4 ± 0.01 mM for mixed type inhibition (Table I). A striking difference between the membrane bound and the solubilized enzyme is seen in their sensitivity to inhibition by free Mn^{2+} ions. A five to ten fold decrease in the K_i values is observed in the solubilized enzyme. A decrease in the K_d of the binding of the free cations which was brought about by a change in conformation due to solubilization might have caused the observed difference. Yet there was only a slight decrease in the K_m values in the solubilized enzyme when compared to the membrane bound form.

Inhibition by free Mg^{2+} ions. In the presence of chloride ions ATPase activity was inhibited by free Mg^{2+} ions with MgATP as substrate. As in the case of Mn^{2+} , the type of inhibition by free Mg^{2+} ions depended on the concentration of the inhibitor (Table I). At low concentrations of free ions mixed type inhibition was observed, while at high concentrations of Mg^{2+} ions uncompetitive inhibition was found. The K_m and the V_{max} of MgATP were only slightly higher than those of MnATP but free Mg^{2+} ions were five to ten fold weaker inhibitors than Mn^{2+} ions. Only small differences were observed between a K_i of 23.3 ± 1.2 mM, and 28.7 ± 0.9 mM for mixed type and uncompetitive inhibition, respectively by free Mg^{2+} ions. However, more than a ten fold decrease in the K_i for Mg^{2+} ions occurred on solubilization of the enzyme. A K_i of 1.93 ± 0.03 mM and an α factor of 3.93 for Mg^{2+} were only slightly higher than the values for Mn^{2+} ions in the solubilized enzyme.

Modulation of kinetic properties by Chloride ions. It is well established that chloride ions stimulate ATPase activity and the coupled pumping of protons (1). Yet the conflicting reports (5-7) with regard to the nature of this effect are understandable in view of the multiple effect of these

stimulate ATPase activity and the coupled pumping of protons (1). Yet the conflicting reports (5-7) with regard to the nature of this effect are understandable in view of the multiple effect of these ions. An attempt was made to separate between the enhancement of the rate of activity and the decrease in the K_i of the free divalent cations. Keeping low free Mg^{2+} ions during measurement of the effect of chloride ions on the rate of activity as a function of MgATP concentrations enabled us to minimize the inhibitory effect caused by the cations. Under these conditions chloride ions changed the V_{max} from 10.9 ± 0.05 to 20.1 ± 0.1 $\mu\text{moles/mg/h}$ causing almost a two fold increase without changing the K_m (Fig. 5). Similar increase in the V_{max} from 9.1 ± 0.3 to 21.3 ± 0.2 $\mu\text{moles/mg/h}$ without and with chloride ions, respectively was observed when the activity was measured in the presence of MnATP and low concentrations of free Mn^{2+} ions (Fig. 6). At higher free Mn^{2+} , chloride ions affected both the apparent K_m and the apparent V_{max} (Fig. 6). It was difficult to make a detailed analysis of such kinetic behavior. It seems however that changes induced by chloride ions both in the K_i and the K_m could cause such kinetic pattern.

In order to determine the affinity of chloride ions to the site which induces the modulation of the kinetic properties of the enzyme the $1/v$ versus $1/Cl^-$ at constant MnATP in the presence of high and low concentrations of free Mn^{2+} was plotted (Fig. 7). The possible multiple effect of chloride ions on both the V_{max} and the K_i for inhibition by free cation is also seen from this analysis. Replot of the apparent K_d gives a dissociation constant of 9.5 ± 0.3 mM for the effect of chloride ions on the change in the V_{max} and the K_i for free cations.

Interaction between chloride and nitrate ions. Nitrate is a specific inhibitor of the vacuolar ATPase which affects both the hydrolytic and the coupled proton pumping activities (1,2). Yet the conflicting reports (6-9) regarding the mode of interaction between chloride and nitrate ions are not surprising in view of the the multiple effects of chloride on the properties of the enzyme. The possibility that the two ions adversely affect the V_{max} of the enzyme was first evaluated. For this reason the effect of chloride ions on the K_i of inhibition by free cations had to be limited by assay of the activity in the presence of low concentrations of free Mn^{2+} ions in the medium. Under such conditions there was a decrease in the inhibition of the rate of ATPase activity by nitrate ions with increased concentrations of chloride ions. A fit of $1/v$ versus $1/Cl^-$ at various concentrations of nitrate indicates a competitive type of interaction between the two anions (Fig. 8). At higher concentrations of free Mn^{2+} mixed type interaction was observed (not shown). From these data dissociation constants for the interaction of 4.5 ± 0.4 mM and 0.5 ± 0.05 mM for chloride and nitrate ions, respectively, were determined. The results point to a possible existence of a common binding site for the two anions.

Nonlinear Kinetics and Sulfite Regulation of the Vacuolar H^+ -ATPase.

As shown in Fig. 9, the yeast vacuolar H^+ -ATPase exhibits nonlinear kinetics in the presence of chloride. Inhibition of the reaction rate during the steady state phase is not prevented by the inclusion of an ATP-regeneration system, indicating that the slower rate is not due to classical

product inhibition. However, sulfite prevents the time-dependent inactivation. Thus, the V-ATPase shows properties similar to those of the F-type and the archaebacterial enzymes.

The effect of substrate concentration on the reaction rate assayed at two different time points is shown in Figs. 10A and B. In panel A, the assay was run for 30 minutes. Note that inactivation at concentrations above 1 mM Sulfite has two effects at the 30 minute time point: below 1 mM MgATP sulfite is inhibitory; above 1 mM MgATP sulfite is stimulatory. However, the major effect of sulfite is to protect against inactivation. Due to the inactivation process the Lineweaver-Burke double reciprocal plot was nonlinear and could not be used to derive K_m or V_{max} . To avoid complications caused by inactivation we examined the kinetics at the 4 minute time point when the reaction is linear. At the early time point sulfite inhibited at all but the highest concentration of MgATP. A Lineweaver-Burke plot of the data indicates that sulfite causes a 5.5-fold increase in the K_m , with only a slight increase in the V_{max} .

Sulfite-Activation and $\Delta \mu H^+$.

Hydrolysis kinetics were carried out using sealed membrane vesicles in the presence of different ionophores. As shown in Fig. 11, sulfite activation was greatest in the presence of gramicidin. Sulfite activation in the absence of ionophores or in the presence of valinomycin was negligible, indicating that sulfite activation is antagonized by $\Delta \mu H^+$, especially by ΔpH . The inhibitory effect of $\Delta \mu H^+$ is in contrast to the F-ATPases, in which $\Delta \mu H^+$ reactivates the ATPase. This is consistent with the opposite function of the V-ATPases, as discussed above.

We also tested whether or not sulfite stimulated proton transport. As shown in Table II, sulfite inhibited proton transport both during the initial phase of the hydrolysis reaction (R1) and during the second phase of the reaction (R2). These and other experiments indicated that sulfite is able to stimulate hydrolysis while actually inhibiting proton transport, suggesting that sulfite induces intrinsic uncoupling or slip.

Control of V-ATPase activity by oxidation-reduction of disulfide bridge in tonoplast vesicles.

The control of activity by formation of a disulfide bond between two cysteines was investigated with the aid of specific oxidizing reagents. Initially we have shown that cupric ions completely inactivated ATPase activity indicating that reduced cysteines are essential for catalytic activity. Reduction of the oxidized enzyme with DTT did not cause a recovery of the activity. It is possible that cupric ions which are strong oxidants oxidized the cysteines to sulfites. Sulfites can not be rereduced as easily as a disulfide bridge formed on the oxidation of a pair of adjacent cysteines. Milder and more specific sulfhydryl oxidant such as oxidized glutathione also inhibited ATPase activity. The inhibition depended on the concentration of the oxidized glutathione (Fig. 12) and caused about 70% inhibition. Time course of the inhibition (Fig. 13) indicated that at the concentration of oxidized glutathione used the inactivation slowed down but did not end even after 1 h indicating that complete inhibition might be reached under optimal

conditions. Yet at any time point of the incubation the inhibition was completely reversed by rereduction with DTT. Reversible inhibition by oxidation and reduction with sulfhydryl reagent indicates that the activity is inhibited by a formation of a disulfide bridge between two essential cysteines in tonoplast ATPase. This interpretation is in harmony with the reported results on site directed mutagenesis of cysteines in V-ATPase from yeasts.

Identification of the essential cysteines which are involved in the regulation of the catalysis by formation of disulfide bond and their subunit location was done by NEM modification. NEM inhibited ATPase activity in tonoplast vesicles (Fig.14) and in the partially purified enzyme (Fig.15). The curve of the dependence of the inactivation on the concentration indicated that at higher than 150 μ M NEM the inhibition tends to increase above the observed 70%. At any given concentration about 70% of the inhibition caused by NEM was prevented by pre oxidation of the enzyme with oxidized glutathione. Since under the experimental conditions only 70% of the activity of the enzyme was inhibited by the oxidation treatment it can be concluded that the cysteines which were involved in the formation of the disulfide bond were modified by NEM.

Identification of the subunit location of the cysteines modified by NEM was done by autoradiography of SDS gel electrophoresis of the enzyme which was treated with radiolabeled reagent. Labeling of tonoplast vesicles with NEM resulted in incorporation of the reagent primarily into a 70k subunit corresponding to the A subunit (Fig.16, lane 2). The labeling intensified as a function of the increase in the concentration of [14 C]NEM from 5 to 150 μ M (Fig. 16, lanes 3-8). Pre oxidation of the enzyme by oxidized glutathione decreased the overall intensity of labeling by NEM (Fig. 17, Lanes 2, 4 and 6). This is in harmony with the finding that peroxidation of the enzyme protects from NEM inhibition. Treatment of the partially purified enzyme at 100 $^{\circ}$ before electrophoresis caused most of the label to stay out of the gel but some of the label remained at the 70k position (Fig. 16, lanes 3 and 4). It is suggested that the label at the 70k region results from binding of NEM to the A subunit of the ATPase and to the tonoplast pyrophosphatase which migrates only slightly slower than the A subunit on the SDS gel. Pyrophosphatase is the upper of the two bands at the 70k region (Fig. 16, lane 2). The upper band disappears on treatment of the partially purified enzyme at 100 $^{\circ}$ before the electrophoresis (data not shown). The upper band is also not seen in the purified enzyme where pyrophosphatase activity is missing. In tonoplast vesicles treated at 100 $^{\circ}$ before electrophoresis there was only slight decrease in the intensity of the 70k band. However, even without the treatment a sizable amount of label did not enter the gel. The suggestion that PPiase is labeled by NEM is supported by the finding that its activity is inhibited by NEM and the inhibition is protected by pre oxidation (data not shown). Labeling of the enzyme in the presence of Mg^{2+} and pyrophosphate greatly decreased the labeling of the pyrophosphatase (Fig. 18, lanes 1 and 2) without affecting the labeling of subunit A. Pre treatment with non radioactive NEM in the

presence of Mg^{2+} and ADP also decreased the intensity of labeling of the pyrophosphatase without a change in the labeling of subunit A (Fig. 18, lanes 3 and 4). ADP and Mg^{2+} protected ATPase activity from inhibition by NEM while pyrophosphate did not (Table III). It can be concluded that the labeled band which remains after treatment at 100° in the partially purified enzyme and in the tonoplast vesicles is the NEM modified A subunit of the ATPase.

Cysteines Required for Catalytic Function

As shown in Fig. 20, the Cys 254 and Cys 261 mutants grew indicating that neither of these cysteines is required for catalytic activity. Although Cys 254 is not conserved, Cys 261 is highly conserved among eukaryotes and is adjacent to the GKTV motif (catalytic site). Thus, it is somewhat surprising that it is not essential for catalytic activity. Note, however, that the double mutant grows rather poorly at pH 7.5, although it clearly grows. This suggests that at least one cysteine is needed in this region for optimal activity.

The growth assays were confirmed by biochemical assays of the mutant enzymes. As shown in Figs. 21 and 22, both nitrate and bafilomycin-sensitive activity was present in the Cys 254 and Cys 261 mutants, as well as in the double mutants. A major finding of this study was that Cys 284 and Cys 538 are required for catalytic activity, as indicated by the failure of the serine substitution mutants to grow at pH 7.5 (see Fig. 20). Cys 284 has been included in the catalytic domain in models based on adenylate kinase (29), whereas Cys 538 is near the carboxy terminus. However, FSBA and 2-azido ATP, two nucleotide analogs, have been shown to bind near this region (29), suggesting that the carboxy terminus is important for catalytic function. We propose that Cys 284 and Cys 538 may participate in a disulfide bond which may be essential for catalytic activity or for proper folding during synthesis.

Identification of the NEM-Binding Site

Eukaryotic V-ATPases have all been shown to be strongly inhibited by the sulfhydryl reagent, N-ethylmaleimide (NEM). F-ATPases, which lack cysteines in the catalytic domain, are relatively insensitive to NEM. Thus we initially proposed that the cysteine located adjacent to the GKTV region (Cys 261) was the site of NEM-binding (15). Recently, Feng and Forgac (28) provided strong evidence that the equivalent residue in the bovine coated vesicle ATPase specifically binds labeled NEM. We tested the NEM-sensitivity of the Cys 254 and Cys 261 mutants to determine which mutation would result in the loss of NEM-binding. As shown in Fig. 23, mutation of Cys 261 to Ser completely eliminated inhibition by NEM, whereas the Cys254->Ser mutation had no effect. The double mutant, as expected, is also insensitive to NEM. This provides additional evidence that Cys 261 is the site of NEM-binding.

Discussion.

Chloride ions decrease the K_i for inhibition by free divalent cations. Magnesium ion is an essential activator of the V-ATPases (1,2). The divalent cation is required for a formation of

MgATP complex which serves as the true substrate (5). In the absence of stimulating anions such as chloride, free divalent cations such as Mg^{2+} or Mn^{2+} ions are poor inhibitors of the activity. Under these conditions only approximate K_i values of 70 mM and 30 mM for Mg^{2+} and Mn^{2+} ions respectively could be obtained because of the weak inhibition. Yet stimulating ion such as chloride are present under physiological conditions. At concentrations of chloride ions which stimulate ATPase activity there is a decrease of 3 to 5 folds in the average K_i to values of 26 mM and 4.6 mM for Mg^{2+} and Mn^{2+} , respectively. The interaction of cations and anions is of special physiological significance in tonoplast ATPase since this pump is involved in regulation of plant cell turgor and in adaptation to salinity.

Two sites for binding of divalent cations. Kinetic analysis indicated that free divalent metal ions interact with the enzyme in a manner which modulates both the K_m and the V_{max} . At low inhibitor concentrations a linear mixed type of inhibition was observed while an uncompetitive type inhibition was observed at higher inhibitor concentrations. Switching from one mode to another could be caused by binding of divalent metal ions to two inhibitory sites. The first is a high affinity site which effects a linear mixed type mode. When the first site is saturated, filling of the second site induces uncompetitive type of inhibition. There is a mechanistic similarity between the two types of inhibitions. In both uncompetitive and mixed type inhibitions there is a change in the V_{max} and the K_m because of the contribution of both the free metal ion and the substrate to the inhibitory complex (Eqs. 3 and 4). The difference between the two modes is due to the fact that in mixed type inhibition binding of the inhibitor to the enzyme changes the affinity of the substrate by an alpha factor ($\alpha > 1$). While in uncompetitive inhibition binding of the substrate induces a change in the affinity of the inhibitor to the enzyme. Based on the two types of inhibition and the different affinity to cations it is suggested that the enzyme contains at least two sites for binding of divalent cations.

Modulation of kinetic properties by chloride ions. The major function of the vacuolar proton ATPase is to generate ΔpH across various membrane organelles (1,2). Symport of chloride ions through a separate channel, which is located in the membranes, is the major mechanism utilized by these systems to determine the size of the ΔpH component of the electrochemical potential generated by this electrogenic proton pump. Modulation of the hydrolytic activity by chloride ions seems to be essential for an efficient control of the overall function of the pump. Chloride ions have a dual effect on ATPase activity. The activity is stimulated by the anions yet the inhibition by free divalent cations is increased in the presence of chloride ions. Therefore the conflicting reports (5-7) with regard to the nature of the effects of chloride ions are understandable. In order to distinguish between the two effects it was necessary to assay the activity in the absence of free divalent cations. Yet since the substrate is a metal-ATP complex which is formed from its components in the solution at any given concentration of the substrate, some free divalent metal ions are present in the solution. By adjusting the relative concentrations

of ATP and of Mg^{2+} it was possible to keep the free metal at a low value at various complex concentrations and to decrease to a minimum its inhibitory effect. Under such conditions it was found that the predominant effect of chloride ions, in the presence of either Mg^{2+} or Mn^{2+} , was to increase the V_{max} without changing the K_m . This was in agreement with the findings of chloride effect in the presence of Mg^{2+} ions on ATPase activity in vacuolar vesicles from beet (5). Interpretation of such data can assume that binding of chloride ions to a location other than the active site induces a higher catalytic rate constant without a change in the binding affinity of the substrate.

The nature of the effect of chloride ions on the interaction of free divalent cations was observed at higher concentrations of the free metal where the inhibition was more pronounced. The change in rate of activity as a function of substrate concentration in the presence of increasing free Mn^{2+} concentrations indicated that chloride affected the apparent K_i and apparent V_{max} . Yet detailed analysis of the kinetic behavior was difficult because of the compound effect of the uncompetitive type of the inhibition by free divalent cations and of the noncompetitive enhancement of the activity by chloride ions with respect to the substrate.

It was possible however to determine the affinity of chloride ion to the site which induces the modulation of the kinetic properties of the enzyme by plotting $1/v$ versus $1/Cl^-$ at constant $MnATP$ in the presence of high and low free Mn^{2+} ions. The possible multiple effect of chloride ions on both the V_{max} and the apparent K_d for Cl^- could also be seen from this analysis. Replot of the apparent K_d gave a dissociation constant of 9.5 ± 0.3 mM for the effect of chloride ions on the change in the V_{max} and on the K_i for free cations. In previous determinations in beet (6) and oat (7) tonoplast vesicles a lower K_d for Cl^- stimulation of ATPase was obtained. An apparent lower K_d would be expected, as can be seen in Fig.7, without taking into consideration the effect of Cl^- on the decrease in the K_i of free divalent cations.

Chloride effect on the transition state conformation of the catalytic subunit. It is puzzling why chloride ions which decreased the K_i for free divalent cations did not decrease the K_m for the substrate which is a complex of divalent cation-ATP. Since the stimulation of the activity was not a result of a change in K_m it could be a result of an increase in the catalytic rate constant. Such an increase could occur when the steady state concentration of the transition state intermediate is increased due to lowering of its K_d by the activator. A lowering of the K_d of the transition state intermediate without a change in the K_m is in harmony with the alternating site binding change hypothesis for catalysis in F-type and V-type ATPases (30). The hypothesis assumes that all three catalytic A subunits alternate in catalysis and in conformation. The catalysis occurs at the subunit to which the substrate is bound with the lowest K_d and very slowly equilibrates with the medium, while binding of the substrate occurs at another subunit having a higher K_d and a fast exchange with the medium. It seems that chloride decreases the K_d for cation binding at the tightly bound substrate on the subunit which is in a transition state conformation and does not

lower than that of ADP complex with divalent cations, an increase in the concentration of the free cations added together with ATP will increase the concentration of cation-ADP complex. When not added, ADP is formed during the hydrolysis of ATP in the reaction. The data recently obtained in our preliminary experiments indicated that MnADP and not free ADP is the effective inhibitor of tonoplast ATPase activity in the presence of chloride ions. Our recent findings on the inhibition of F-ATPase from chloroplasts (31) and the nonlinear kinetics presented in this report also indicate that divalent cation-ADP might be the inhibitor of the activity.

Nonlinear Kinetics and Sulfite Regulation of the Vacuolar H⁺ATPase.

We propose that sulfite-induced protection against inactivation at the later time points can also be explained by an increase in the K_m for nucleotide. At low MgATP concentrations, when MgATP is limiting, sulfite inhibits the activity by increasing the K_m for the substrate. At high MgATP concentrations, when product release becomes limiting, increasing the K_m stimulates the activity. Inactivation is prevented by increasing the rate constant for the release of the nucleotide bound to the catalytic site. The longer the time that the product remains at the catalytic site, the greater the chance that the enzyme will become inactivated.

This model differs somewhat from that proposed by Boyer and colleagues for the F₁-ATPases (11, 12). In their model, sulfite increases V_{max} and has no effect on K_m . In other words, sulfite promotes the release of tightly-bound nucleotide from the inactive form of the enzyme, thereby reactivating it. Whether this represents a basic difference between the F- and V-type ATPases remains to be seen.

Control of ATPase activity by formation of disulfide bridge between essential cysteines in the A subunit. The role of highly conserved cysteine residues in the catalytic site was investigated by constructing a series of Cys → Ser mutants in A subunit of yeast V-ATPase. Changing Cys261 to serine did not affect catalytic activity but completely eliminated inhibition by NEM. Thus Cys261, which is located next to the nucleotide-binding motif "GKT", is the site of NEM binding. Changing either Cys284 or Cys538 to serine resulted in a loss of activity, demonstrating that they are essential. An important control mechanism induced by oxidation-reduction of a disulfide bridge in the A subunit is indicated from the experiments with thiol specific reagent. Although the cysteine in tonoplast ATPase which is equivalent to cys261 in yeast is not an essential residue its modification with NEM causes a disturbance which partially inhibits the activity. It is possible that cyst261 takes part in formatin of a disulfide bridge which controls the activity. Oxidation of this cysteine and the resulted formation of the disulfide bridge prevents the modification of cys261 by NEM.

An important byproduct of our experiments is the finding of inhibition and modification of the H⁺-pyrophosphatase by NEM in tonoplasts. This finding which was independently found by Rea et al (32) indicated that cysteine might be an essential residue in the catalysis of pyrophosphatase activity. In addition we have shown that pyrophosphatase activity is controlled

H⁺-pyrophosphatase by NEM in tonoplasts. This finding which was independently found by Rea et al (32) indicated that cysteine might be an essential residue in the catalysis of pyrophosphatase activity. In addition we have shown that pyrophosphatase activity is controlled by oxidation reduction of a disulfide bridge. It seems therefore that the thiol status is an important mechanism in the control of the two major electrogenic proton pumps in tonoplasts.

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Legends to Figures.

Fig.1. The dependence of ATPase activity in tonoplast vesicles on Mg^{2+} and Mn^{2+} ions. The activity was assayed in a medium containing: 30 mM Tris-Mes, pH 7.5, 60 mM KCl, 2mM ATP, 15 μ g tonoplast vesicles protein, 0.5 mM NaN_3 and the indicated concentrations of $MgSO_4$, ($\square$) or $MnSO_4$ (O) at 30° for 30 min. The released Pi was determined spectrophotometrically as described under Experimental procedure.

Fig.2. MnATP as the true substrate of ATPase activity in tonoplast vesicles. The activity was assayed with various concentrations of MnATP, 60 mM KCl and 4 mM (O), 8 mM (Δ) and 16 mM ($\square$) free Mn^{2+} ions. A dissociation constant of 16 mM for the complex was used in the calculations.

Fig. 3. Uncompetitive inhibition of tonoplast ATPase activity by free Mn^{2+} ions. A double reciprocal plot of the data in Fig. 3. A replot of the apparent $1/V_{max}$ versus various concentrations of free Mn^{2+} (inset).

Fig. 4. Mixed type inhibition of tonoplast ATPase activity by low concentrations of free Mn^{2+} ions. The activity was measured at various concentrations of MnATP, 60 mM KCl and in the presence of 0.3 mM (O) and 2 mM (Δ) free Mn^{2+} ions. The conditions were as indicated under Experimental procedure. A double reciprocal plot of the rate of activity versus the concentration of MnATP indicates a mixed type inhibition (inset).

Fig.5. The non interacting nature of the stimulation of ATPase activity in tonoplast vesicles by KCl. A double reciprocal plot of the rate of activity versus the concentration of MgATP in the presence of 7mM free Mg^{2+} ions. The activity was measured in the absence (O) and in the presence ($\square$) of 50 mM KCl.

Fig.6. Stimulation of tonoplast ATPase activity is not caused by direct interaction between KCl and MnATP. ATPase activity was measured in the presence of various concentration of MnATP,

measured in the presence of 2 mM MnATP and 0.3 mM (O) or 3 mM(□) free Mn^{2+} ions at various concentrations of KCl. A double reciprocal plot of the rate of activity versus KCl is presented.

Fig. 8. Competition between Cl^- and NO_3^- ions on the effect on ATPase activity in tonoplasts. The activity was measured in the presence of 2 mM MnATP, 0.3 mM free Mn^{2+} ions at various concentrations of KCl with 0 mM (O), 0.5 mM (□) and 2 mM NO_3^- (A). The data are presented as double reciprocal plot of activity versus concentrations of KCl. The activity was assayed with 2 mM MnATP and 0.3 mM free Mn^{2+} ions.

Fig.9.. Time course of ATP hydrolysis by the yeast V-ATPase. Open triangles = 100 mM KCl; closed circles = 100 mM KCl plus an ATP regeneration system; open circles = 100 mM K_2SO_3 .

Fig. 10. Effect of increasing substrate concentration on the activity of the yeast V-ATPase. A. Assayed at 30 minutes; B. Assayed at 4 minutes. Open circles = 100 mM KCl; closed circles = 100 mM K_2SO_3 . Inset shows Lineweaver-Burke plot.

Fig.11. Effects of ionophores on the ATP hydrolysis time course of sealed vacuolar membrane vesicles from yeast. Panel A. a = gramicidin plus sulfite (100 mM); b = chloride (100 mM); c = sulfite (100 mM); d = gramicidin plus chloride. Panel B. e = gramicidin plus sulfite; f = nigericin plus sulfite; g = sulfite; h = valinomycin plus sulfite; i-k = sulfate controls (not discussed). Note that collapsing the pH gradient (gramicidin or nigericin) gives the highest activity in the presence of sulfite. This suggests that sulfite stimulation is antagonized by delta pH.

Fig.12. The effect of oxidized glutathione on tonoplast ATPase activity. Tonoplast vesicles containing 20 μg protein were pretreated and then incubated in a medium containing various concentrations of oxidized glutathione for 1 h at 25°. ATPase activity was assayed by addition of reaction medium containing MgATP to a final volume of 0.5 ml as described under Experimental Procedure.

Fig. 13. Time course of the effect of glutathione on tonoplast ATPase activity. Samples of tonoplast vesicles were incubated in 20 mM oxidized glutathione in a total volume of 7 μl as described under Fig. 12. ATPase activity was assayed at the indicated time intervals with 2.5 mM (□) and without (O) DTT in a total volume of 0.5 ml. Vesicles which were pre incubated without glutathione were assayed without (O) or with (□) 2.5 mM DTT.

without glutathione were assayed without (●) or with (■) 2.5 mM DTT.

Fig. 14. The effect of glutathione on the inhibition of tonoplast ATPase by NEM. Treated vesicles were pre incubated for 2 h at 25° with (●) or without (○) 10 mM oxidized glutathione. Samples of 10 μ l were added to a solution containing the indicated concentrations of NEM in a total volume of 20 μ l and incubated for 10 min at 25°. The reaction was terminated by the addition of 10 mM DTT. ATPase activity was assayed in a total volume of 0.5 ml.

Fig. 15. The effect of oxidized glutathione on the inhibition of partially purified ATPase by NEM. Partially purified tonoplast ATPase 5 to 10 μ g protein was treated and incubated with (●) and without (○) oxidized glutathione and with NEM as described under Fig. 14.

Fig. 16. Labeling of partially purified tonoplast ATPase by [14 C]NEM. The enzyme (15 μ g protein) was incubated with 5 μ M, 10 μ M, 20 μ M, 40 μ M, 80 μ M and 150 μ M of [14 C]NEM, lanes 2 to 7. Subunits were separated on SDS gel electrophoresis and the gel exposed to x-ray film as described under Experimental procedure. Molecular weight markers are on lane 0.

Fig. 17. The effect of oxidized glutathione on the labeling of ATPase by [14 C]NEM. Samples of partially purified ATPase, lanes 1 to 4 and of tonoplast vesicles, lanes 5 and 6 were incubated with [14 C]NEM and separated by SDS gel electrophoresis. Samples on lane 2, 4 and 6 were treated with oxidized glutathione prior to incubation with NEM. The protein samples on lanes 3 and 4 were incubated for 2 min at 100° before electrophoresis.

Fig. 18. The effect of pre treatment with NEM and MgADP on the labeling of partially purified ATPase with [14 C]NEM. Samples of partially purified ATPase were pre incubated with 0.1 mM NEM in the presence of 5.4 mM ADP and 0.43 mM MgSO₄ for 10 min at 21°. Following the pre incubation the samples were passed over a Sephadex G-50 column, incubated with [14 C]NEM and separated by SDS gel electrophoresis as described under Fig. 13. In lanes 1 and 2, 1.3 mM MgSO₄ and 0.3 mM pyrophosphate were added to the medium during the incubation with [14 C]NEM. The samples in lanes 5 and 6 were treated with [14 C]NEM without pre treatment with NEM and MgADP. The samples in lanes 2, 4 and 6 were incubated for 2 min at 100° before electrophoresis.

Fig. 19. Alignments of the glycine-rich nucleotide-binding loop of seven eukaryotic V-ATPase A subunits (A) and the carboxy terminus region of seven eukaryotes and four archaebacteria (B).

The highly conserved cysteines are boxed. Note that Cys 261 is adjacent to the conserved "GKT" motif and Cys 284 is downstream from this region. Cys 538 is near the carboxy terminus. Yeast = Sc69.

Fig. 20. Comparison of growth at pH 5.5 vs pH 7.5 for three of the cysteine mutants. WT = wild type; C1S = Cys 254 -> Ser; C1C2 = double mutant (Cys 254, 261 -> Ser); C2S = Cys 261 -> Ser; C3S = Cys 284 -> Ser; Con = V-ATPase null mutant. Note that the Cys 254 and Cys 261 mutants, as well as the double mutant, grow at both pH 5.5 and 7.5, indicative of active V-ATPases. The Cys 284 mutant, however, fails to grow at pH 7.5. Growth of the Cys 538-> Ser mutant (not shown) was identical to Cys 284.

Fig. 21. Effect of nitrate on the ATPase activities of the cysteine mutants. wt = wild type; #24 = wild type gene expressed on plasmid YPNTR; #7 = Cys 254 -> Ser; #42 = Cys 261 -> Ser; #59 = double mutant. Other numbers indicate other mutants not discussed.

Fig. 22. Effect of bafilomycin on the ATPase activities of the cysteine mutants. wt = wild type; #24 = wild type gene expressed on plasmid YPNTR; #7 = Cys 254 -> Ser; #42 = Cys 261 -> Ser; #59 = double mutant. Other numbers indicate mutants not discussed.

Fig. 23. Effect of NEM (N-ethylemaleimide) on the ATPase activities of the cysteine mutants. wt = wild type; #24 = wild type gene expressed on plasmid YPNTR; #7 = Cys 254 -> Ser; #42 = Cys 261 -> the cysteine mutants. wt = wild type; #24 = wild type gene expressed on plasmid YPNTR; #7 = Cys 254 -> Ser; #42 = Cys 261 -> Ser; #59 = double mutant.

Tables

Table I

Kinetic constants for the inhibition of ATPase activity by free Mg^{2+} and Mn^{2+} ions in tonoplast vesicles and in partially purified enzyme.

The kinetic parameters were calculated as described under Experimental Procedure using similar data to those under Figs 2-4. V_{max} values are given at $\mu\text{moles/mg/h}$.

Inhibition	Substrate	$K_m(\text{mM})$	$K_i(\text{mM})$	α	V_{max}
Tonoplast vesicles					
Uncompetitive	MnATP	0.3 ± 0.040	5.2 ± 0.3	1.0	11.5 ± 1.1
Mixed	MnATP	0.31 ± 0.04	3.9 ± 0.2	3.7	10.7 ± 1.0
Uncompetitive	MgATP	0.45 ± 0.02	28.7 ± 1.9	1.0	15.6 ± 1.3
Mixed	MgATP	0.75 ± 0.03	23.3 ± 1.3	2.7	15.7 ± 1.3
Partially purified enzyme					
Uncompetitive	MnATP	0.13 ± 0.01	0.91 ± 0.01	1.0	11.5 ± 1.1
Mixed	MnATP	0.17 ± 0.01	0.42 ± 0.01	2.0	10.7 ± 1.0
Mixed	MgATP	0.23 ± 0.01	1.98 ± 0.03	3.9	15.7 ± 1.3

Table II

Effect of Sulfite On Proton Pumping During R_1 and R_2

	<u>100 mM KCl</u>		<u>100 mM K₂SO₃</u>	
	Initial Rate %/Min.	% Total Quench After 5 Min.	Initial Rate %/Min.	% Total Quench After 5 Min.
R_1	83.9	33.2	61.8	16.6
R_2	64.5	16.6	12.7	12.0

Table III

The effect of MgADP on inhibition of ATPase activity by NEM

Samples of partially purified ATPase were preincubated with or without 0.1 mM NEM in the presence or absence of 5.4 mM ADP and 0.43 mM MgSO₄ for 10 min at 21°. Following the pre incubation the samples were passed over a Sephadex G-50 column and incubated with [¹⁴C]NEM.

Additions			ATPase Activity (%)
Pre incubation	Incubation	Reaction	
None	None	None	100
None	NEM	None	54
NEM,MgADP	None	None	109
NEM,MgADP	NEM	None	38
NEM,MgADP	None	MgPPi	98
NEM,MgADP	NEM	MgPPi	24.5

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Fig. 9

Fig. 10

Fig. 1

Fig. 12

Fig. 13

Fig. 14

Fig. 15

Fig. 16

Fig. 17

Fig. 18

A

Glycine Rich Loop Of Eukaryotic 70's

Bovine
Insect
Carrot
Candida
Yeast
Fission Yeast
N.crassa

P C V Q G G T T A I P G A F G C G K T V I S Q S L S K Y S N S D V I I Y V G C G E R
P C V Q G G T T A I P G A F G C G K T V I S Q A L S K Y S N S D V I I Y V G C G E R
P S V L G G T T C A I P G A F G C G K T V I S Q A L S K Y S N S D T V V Y V G C G E R
P C V Q G G T T C I P G A F G C G K T V I S Q S L S K F S N S D V I I Y V G C G E R
P C V Q G G T T C I P G A F G C G K T V I S Q S L S K Y S N S D A I I Y V G C G E R
P C V Q G G T T A I P G A F G C G K T V I S Q S L S K Y S N S D L I V Y V G C G E R
P S V Q G G T V A I P G A F G C G K T V I S Q S V S K F S N S D V I V Y V G C G E R

261

284

B

Bp69
Ms69
Dc69
Ct69
Sc69
Sp69
Nc69
Has69
Meb69
Tht69
Sua69

FLQONGYTPYDRFCPEFYKTVGMLSNMIAFYDMARRAVETTAQSD-NKITWSIIREHMGEILYKLSS 584
FLQONSYSYDRFCPEFYKTVGMLKNIIISFYDMSRHAVESTAQSD-NKVTWNVIRDAMGNVLYQLSS 583
YLAQNAFTPYDKFCPEFYKSVWMMRNIIHFYNLANQAVERGAGMDGQKISYTLIKHRLGDLFYRLVS 587
FLQONGYSYDAFCPEIWKTFTDMMRAFIISYDEAQKAIANG-----AQWSKLAESTSDVKHAVSS 584
FLQONGYSTYDAFCPEIWKTFTDMMRAFIISYHDEAQKAVANG-----ANWSKLADSTGDKHAVSS 584
FLQONGYSYDRCCPLYKTYHMMRNMIAYYTKAKSAVETG-----SVPWSKIKESTSDIFYELTS 586
FLQONGYSYDQFCPEIWKTFTDMMKLMGFHDEAQKAIQAG-----QNWNVREATQDLQAQLKS 573
WLQQNALHDVDRYCPPEKTYAILSGIKTLHEESFEALDAG-----VPVEETSIDAAPRLNRL- 558
FLQONAFHPVDAYSFPDQQYKILKAIMKWGDAAMDALKSG-----VPVTEI IKLESKNVLAKVK 552
FLQONAYHEVDAYCSMKKAYGIMKMILAFYKEAEEAAIKRG-----VSIDEILQLPVLERIGRA- 551
FLKENAYDDIDAFSSPQKQVRIMRLTIYFYNQSQDILISKG-----VPLKKILDKVGPIEPIIR 559

834

Fig. 20

Fig. 21

Fig. 22

Fig. 23

F. Cooperation. In the early stages of the project the cooperation was based mainly on exchange of information and experience in the search for a reliable plant source for the isolation of tonoplast vesicles and for the purification of the ATPase. To facilitate interaction and foster greater communication the two P.I.s spent a total of five months working together in the laboratory of Dr. Nathan Nelson. A major objective was to learn molecular techniques and to plan future experiments. A decision was made to utilize yeast as a model system for molecular analysis of the catalytic subunit. Preliminary experiments were also carried out together using FPLC to purify the Mung bean and the yeast vacuolar ATPase. Discussions were continued at a Gordon Conference on Bioenergetics. Exchange of on going information from the experiments on site directed mutation of cysteines and the work on the oxidation control and NEM modification proved to be crucial for the conductance of a successful project. Antibodies to the A subunit raised at Santa Cruz were mailed to Tel Aviv.

G. Evaluation of research achievements.

Cell expansion in plants is driven by the osmotic accumulation of water in the central vacuole, which occupies over 90% of the cell volume. Solute transport in vacuoles is an active process powered primarily by the vacuolar ATPase. Hence understanding the mechanism of the vacuolar ATPase is an important objective for increasing plant growth and crop productivity. Under physiological conditions the pump works in an environment which contains various anions and divalent cations. The composition of ions changes with environmental changes such as salinity, and growth stages. Chloride ions at concentrations which are present in the cell are known to stimulate the activity of the pump. We confirmed that the stimulation is due to increase in V_{max} , however we discovered that the anions also induce an inhibition by divalent cations. The inhibition changes depending on the type of the cation thus the activity of the pump responds and is being adjusted in accordance with the type of the divalent cation. The regulation is fine tuned as there are two cation binding sites which are distinguished by their affinity and by the type of inhibition which they induce.

We found indications that the inhibition might be caused not by direct binding of the cation but by binding of a cation-ADP complex. The findings were in harmony with the suggestion that catalysis occurs at the subunit to which the substrate is bound with the lowest K_d and very slowly equilibrates with the medium, while binding of the substrate occurs at another subunit having a higher K_d and a fast exchange with the medium. It seems that chloride decreases the K_d for cation binding at the tightly bound substrate on the subunit which is in a transition state conformation and does not alter the K_d at the subunit which binds the substrate loosely, thus the K_m is unchanged. Other anions such as oxyanions stimulated the activity without increasing the inhibition by cation-ADP. It is possible that when they are present in the cytoplasm, under specified physiological conditions, a more active pump is favored.

The discovery that V-ATPases, like F-ATPases, have nonlinear kinetics has important implications for living plant cells. It implies that at steady state there is an equilibrium between active and inactive forms of the enzyme, and that stimulatory ions such as sulfite can shift the equilibrium to the active form. Regulation of this sort may be important for plant growth, which is sometimes interrupted by adverse conditions such as water stress. Resumption of growth presumably requires a reactivation process. It may be possible in the future to alter the V-ATPase in a way that enhances the reactivation process, thereby speeding recovery and increasing crop yields under favorable conditions.

We have established that the catalytic subunit contains two essential cysteines which may form disulfide bonds. In addition, we have shown that Cys261 is the site of inhibition by NEM. Since Cys261 is not essential for catalytic activity, although it is highly conserved in eukaryotes, Cys 261 may play a role in the regulation of the enzyme. Altering this cysteine in the higher plant vacuolar ATPase may make the enzyme less susceptible to oxidative inhibition *in vivo*. It will be interesting to determine whether this would result in a more active ATPase and a higher growth rate for the plant mechanism of anion stimulation of the vacuolar ATPase.

H. Publications

Carmeli, C. and Lifshitz, Y. (1993) Modulation of ATPase activity by divalent cations and anions in the membrane bound and the soluble tonoplast H⁺-ATPase from Mung beans.

J. Biol. Chem. (Submitted).

Carmeli, C. and Lifshitz, Y. Control of tonoplast ATPase activity by oxidation-reduction of disulfide bond. (In preparation).

Kibak, H., Van Eeckhout, D., Cutler, T., Taiz, S.L., and L. Taiz. (1993). Sulfite both stimulates and inhibits the yeast vacuolar H⁺-ATPase. J. Biol. Chem. (In press)

Taiz, L., Nelson, H., Maggert, K., Morgan, L., Taiz, S.L., Yatabe, B., and Nelson, N. (1993) The role of conserved cysteines in the A subunit of the yeast vacuolar H⁺-ATPase. [In preparation]