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An essential arginine residue in vacuolar H^+-ATPase purified from etiolated mung bean seedlings

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Abstract. Treatments of the tonoplast ATPase purified from mung bean seedlings (*Vigna radiata* L.) with guanidino modifiers, phenylglyoxal and 2,3-butanedione, caused a marked loss of the ATP hydrolysis activity and proton translocation in a concentration-dependent manner. Kinetic analysis yielded first order rate constants, k_2, of 0.416 and 0.227 s^{-1} and steady-state dissociation constants, K_i, of 19.3 and 24.2 mM for phenylglyoxal- and butanedione-inhibition of vacuolar H^+-ATPase, respectively. The reaction order of phenylglyoxal- and butanedione-inhibition was calculated to be 0.94 and 0.89, respectively, suggesting that at least one arginine residue of vacuolar H^+-ATPase was modified by both reagents. Lineweaver-Burk plots showed that the mode of inhibition of vacuolar H^+-ATPase by both modifiers is competitive. Mg-ATP, the physiological substrate of vacuolar H^+-ATPase, but not its analogs, exerted preferentially partial protection against phenylglyoxal and butanedione, indicating that the arginine residue involved in the inhibition of enzymatic activity may be located at or near the active site and directly participate in the binding of the substrate.

Keywords: Chemical modification; H^+-ATPase; Mung bean; Proton translocation; Tonoplast.

Abbreviations: BD, 2,3-butanedione; **BZ-ATP,** 3-O-(4-benzoyl) benzoyladenosine 5'-triphosphate; **DCCD,** N,N'-dicyclohexyl carbodiimide; **DIDS,** 4,4'-diisothiocyanostilbene-2,2'disulfonate; **NBD-Cl,** 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; **NEM,** N-ethylmaleimide; **PGO,** phenylglyoxal.

Introduction

Tonoplast H^+-ATPase of higher plants plays a vital role in the secondary active transport of sugars, ions, and metabolites (Sze, 1985; Sze et al., 1992). Tonoplast H^+-ATPase is discretely stimulated by Cl^- and inhibited by NO_3^-, DIDS[2], NEM, NBD-Cl, and DCCD (Sze, 1985; Sze et al., 1992). Molecular mass of tonoplast H^+-ATPase from higher plants was estimated to be 400–600 kDa (Parry et al., 1989; Matsuura-Endo et al., 1990). Vacuolar H^+-ATPase contains seven to nine polypeptides upon SDS-PAGE with two major bands of molecular masses of 65–80 kDa (A subunit) and 55–65 kDa (B subunit), and a 16-kDa protein (DCCD-binding proteolipid). The A subunit of vacuolar H^+-ATPase is implicated as the catalytic site (Randall and Sze, 1987; Bowman et al., 1986), while the B subunit probably plays a regulatory role (Bowman et al., 1986; Manolson et al., 1985). The DCCD-binding proteolipid subunit might be involved in the proton translocation and is preferentially labeled by radioactive DCCD (Mandala and Taiz, 1986; Manolson et al., 1985; Randall and Sze, 1987).

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The cDNA sequences and consequently their deduced amino acid sequences of A subunit from tonoplast H^+-ATPases of carrot roots (Zimniak et al., 1988), mung bean (Chiu et al, 1995), cotton (Wilkins, 1993), and *Neurospora crassa* (Bowman et al., 1988) were recently obtained. Several essential amino acid residues involved in the catalytic activity of vacuolar H^+-ATPases from coated vesicles and yeasts were investigated by substrate analogs (Vasilyeva and Forgac, 1996; Zhang et al., 1995; Feng and Forgac, 1994; Manolson et al., 1985; Mandala and Taiz, 1986) and site-directed mutagenesis (Liu et al., 1997; Liu et al., 1996). For instance, the sensitivities of vacuolar H^+-ATPase to NEM and NBD-Cl imply the possible presence of cysteine and/or tyrosine residues at the active site (Manolson et al., 1985; Mandala and Taiz, 1986). Further mutation analysis reveals the roles of glycine and glutamate residues in the stability and the catalytic activity of vacuolar H^+-ATPase (Liu et al., 1997). Our recent work also indicates, using the substrate analog dialdehyde derivative of ATP, the A subunit of vacuolar H^+-ATPase contains a lysine residue essential to the enzymatic activity (Chow et al., 1992; Tzeng et al., 1992). In spite of these results, further efforts are required to identify other amino acid residues significant to the activity of vacuolar H^+-ATPase and to elucidate their roles in the enzymatic mechanism.

Arginine residues were shown to participate in catalytic activities, substrate binding, and maintenance of enzyme conformation of F- and P-type H^+-ATPases (Vallejo, 1973; Kasamo, 1988). However, no evidence directly reveals whether arginine residues are also involved in the catalytic activity of purified V-type H^+-ATPase. Phenylgloxal (PGO) and 2,3-butanedione (BD) have been widely used to identify arginine residues and determine their roles in enzymatic activity of various enzymes (Riordan, 1973; Kuo and Pan, 1990). In this study, we demonstrated the presence of an arginine residue may be involved in the inhibition of ATP hydrolysis and H^+-translocation by PGO and BD. Kinetic analysis indicates that modification of one mol arginine/mol ATPase is sufficient to inhibit the enzymatic activity of vacuolar H^+-ATPase.

Materials and Methods

Plant Materials

After soaking in tapwater for 24 h, seeds of *Vigna radiata* L. (mung bean) were germinated at room temperature in the dark. Hypocotyls of 4-day old etiolated seedlings were excised, chilled, and then used as starting materials.

Isolation of Tonoplast Vesicles

Tonplast vesicles were prepared by a floating method of Matsuura-Endo et al. (1990) with minor modifications. Chilled hypocotyls were ground with a Waring blender in a homogenization medium containing 50 mM Mops/KOH (pH 7.6), 0.25 M sorbitol, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM potassium metabisulfite, and 1.5% (w/v) polyvinylpyrolidone. The homogenate was filtered through four layers of cheesecloth and subjected to differential centrifugation at 3,600 *g* for 10 min and 150,000 *g* for 20 min. The pellet was suspended in a suspension solution containing 10 mM potassium phosphate (pH 7.8), 0.3 M sucrose, 1 mM EGTA, and 2 mM dithiothreitol. The suspension was overlaid in a medium containing 5 mM Mops/KOH (pH 7.3), 0.25 M sorbitol, 1 mM EGTA, and 2 mM dithiothreitol. After centrifugation at 120,000 *g* for 40 min, the interface portion was collected and diluted with 5 mM Mops/KOH (pH 7.3), 0.25 M sorbitol, 1 mM EGTA, and 2 mM dithiothreitol. The suspension was then centrifuged at 150,000 *g* for 20 min, and the resulting white pellet (vacuolar-membrane vesicles) was suspended in a medium containing 10 mM Tris/Mes (pH 7.5), 0.25 M sorbitol, 2 mM dithiothreitol, and the protein concentration of 3 mg/ml.

Purification of Vacuolar H^+-ATPase

Vacuolar H^+-ATPase was purified from resealed tonoplast vesicles according to the method of Matsuura-Endo et al. (1990) with minor modifications. Triton X-100 and NaCl were added dropwise to the membrane fraction to final concentrations of 5% (w/v) and 0.1 M, respectively. The suspension was stirred at 4°C for 30 min and centrifuged at 150,000 *g* for 30 min. The pellet was suspended in Buffer A [20 mM Tris/acetate (pH 7.5), 1 mM EGTA, 1 mM dithiothreitol, 2 mM $MgCl_2$, and 20% (v/v) glycerol]. Lysophosphatidylcholine was added to the suspension at a final concentration of 4 mg/ml. The mixture was stirred for 30 min at 25°C and centrifuged at 150,000 *g* for 40 min. The supernatant was applied onto a Mono Q HR5/5 anion-exchanger FPLC column (0.5 × 5 cm) pre-equilibrated with running buffer [Buffer A + 0.1% (w/v) Triton X-100] at room temperature. The sample was eluted at a flow rate of 0.5 ml/min with NaCl gradient (40 ml) from 0 to 0.5 M. Fractions (1 ml) were collected and aliquots (20 µl) were withdrawn for enzyme assay. Fractions with highest ATPase activity were pooled and stored at -70°C for further studies.

Enzyme Assay and Protein Determination

Activities of tonoplast H^+-ATPase were measured in a 0.5 ml reaction solution. The mixture contained 30 mM Tris-HCl (pH 7), 3 mM $MgSO_4$, 50 mM KCl, 3 mM ATP, 0.5 mM sodium azide, 0.1 mM sodium vanadate, 0.1 mM ammonium molybdate, 40 µg phosphatidylcholine, and 12–20 µg of protein. After 30 min incubation at 37°C, the reaction was stopped by adding a solution containing 1.7% (w/v) ammonium molybdate, 2% (w/v) SDS, and 0.02% (w/v) ANSA (1-amino-2-naphthol-4-sulfonic acid) at room temperature. The released P_i was measured spectrophotometrically (Wang et al., 1989).

Protein concentration was determined according to a modified Lowry method using bovine serum albumin as the standard (Larson et al., 1986).

Measurement of Proton Translocation

Proton translocation was measured as described previously using fluorescence quenching of Acridine Orange (Wang et al., 1989). The reaction mixture contained 5 mM Tris-HCl (pH 7), 250 mM sorbitol, 3 mM ATP, 3 mM $MgSO_4$, 50 mM KCl, 0.5 mM NaN_3, 0.1 mM Na-vanadate, 0.1 mM ammonium molybdate, 5 µM Acridine Orange, and 10–20 µg/ml membrane protein. The fluorescence quenching was initiated by adding 3 mM $MgSO_4$. The ionophore gramicidin (2 µg/ml) was added at the end of each assay.

Modification of ATPase with Modifiers

Vacuolar H^+-ATPase was modified with various concentrations of PGO and BD in 50 mM Mops-KOH (pH 7) for time periods indicated at room temperature. At the end of incubation the mixture was diluted 25 fold with assay solution as described above, and enzyme assay started immediately.

Kinetic Analysis

Lineweaver-Burk plots were obtained conventionally and values of K_m and V_{max} were thus determined directly from intercepts. The $t_{1/2}$ values, time required for 50% inhibition of activity, at various concentration of modi-

fiers were measured according to semi-logarithmic plots of percent residual activity versus time as described by Levy et al. (Levy et al., 1963). The first order rate constant, k_2, and reaction order (n) with respect to the modifiers were determined from double-logarithmic plots of the apparent rate constant, K_{obs}, versus the concentration of modifiers as follows (Carlson, 1984; Rakitzis, 1978, 1984):

(1) $\log K_{obs} = n \log [I] + \log k_2$

where K_{obs} is the apparent reaction rate constant; k_2 is the first order rate constant for inactivation; and [I] is concentration of modifers. The steady-state dissociation constant of inhibition, K_i, was estimated from the slope of the plot according to the equation 2 (Carlson, 1984):

(2) $$\frac{1}{K_{obs}} = \frac{K_i}{k_2} \times \frac{1}{[I]} + \frac{1}{k_2}$$

Chemicals

PGO and BD were obtained from Sigma and ATP from Merck. All other chemicals were of the highest grade available from commercial sources.

Results and Discussion

Inactivation of ATPase by Phenylglyoxal and 2, 3-Butanedione

Incubation of vacuolar membrane with BD and PGO resulted in a progressive loss of ATP hydrolysis in a concentration-dependent manner (Figure 1A). The half maximal inhibition of membrane-bound H^+-ATPase by BD and PGO occurred approximately at 35 and 9 mM, respectively. Similarly, I_{50} values (concentration of modifiers at which 50% activity of control is inhibited) of BD- and PGO-inhibition of purified vacuolar H^+-ATPase were obtained (Figure 1B). In parallel experiments, BD- and PGO-inhibition of ATP-mediated H^+-translocation into vacuole as measured by fluorescence quenching of Acridine Orange was also observed (Figure 2). The rate of proton-translocation was markedly decreased following the treatment of tonoplast vesicles with BD and PGO, respectively, at various concentrations. However, neither modifier likely exerts any significant effect on the membrane leakage, since the coupling efficiency (expressed as the ratio of proton translocation/enzymatic acitvity) of the vacuolar H^+-ATPase (data not shown) was not changed after the addition of inhibitors. Concentration for half maximal inhibition of proton translocation by BD is similar to that for enzymatic activity of membrane-bound H^+-ATPase. Moreover, a larger I_{50} value was obtained for inhibition of H^+-translocation than ATP hydrolysis activity of vacuolar membrane by PGO. These results indicate that ATP-hydrolysis and H^+-translocation of vacuolar H^+-ATPase might be indirectly linked *in situ*. Similar phenomena were observed in F-type ATPase (Tu et al., 1990) and sulfhydryl and lysine modification of vacuolar H^+-ATPase (Chow et al., 1992). Moreover, purified H^+-ATPase is approximately fourfold more sensitive to guanidino modifiers than membrane-bound enzyme. The relatively higher sensitivity for purified H^+-ATPase excludes the side effect of isolated membranes which might be a mixture of "right-side out" and "inside-out" orientated vesicles. It is suggested that membranes might play a significant role in blocking the access of inhibitors to the target amino acid residues in vacuolar H^+-ATPase.

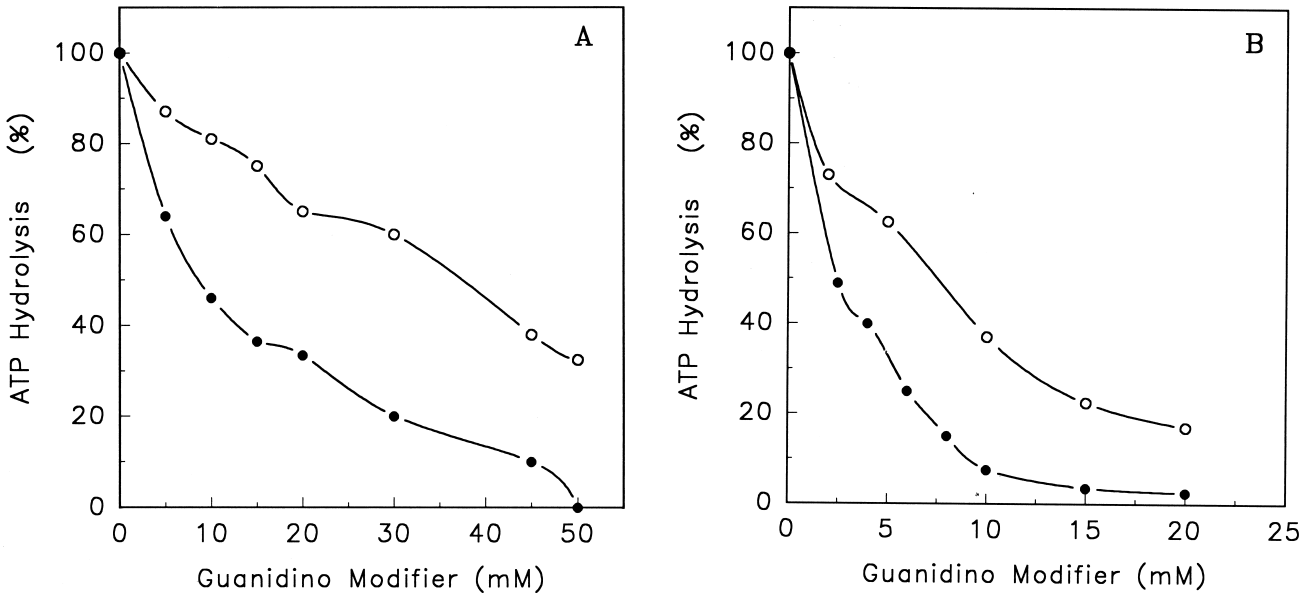
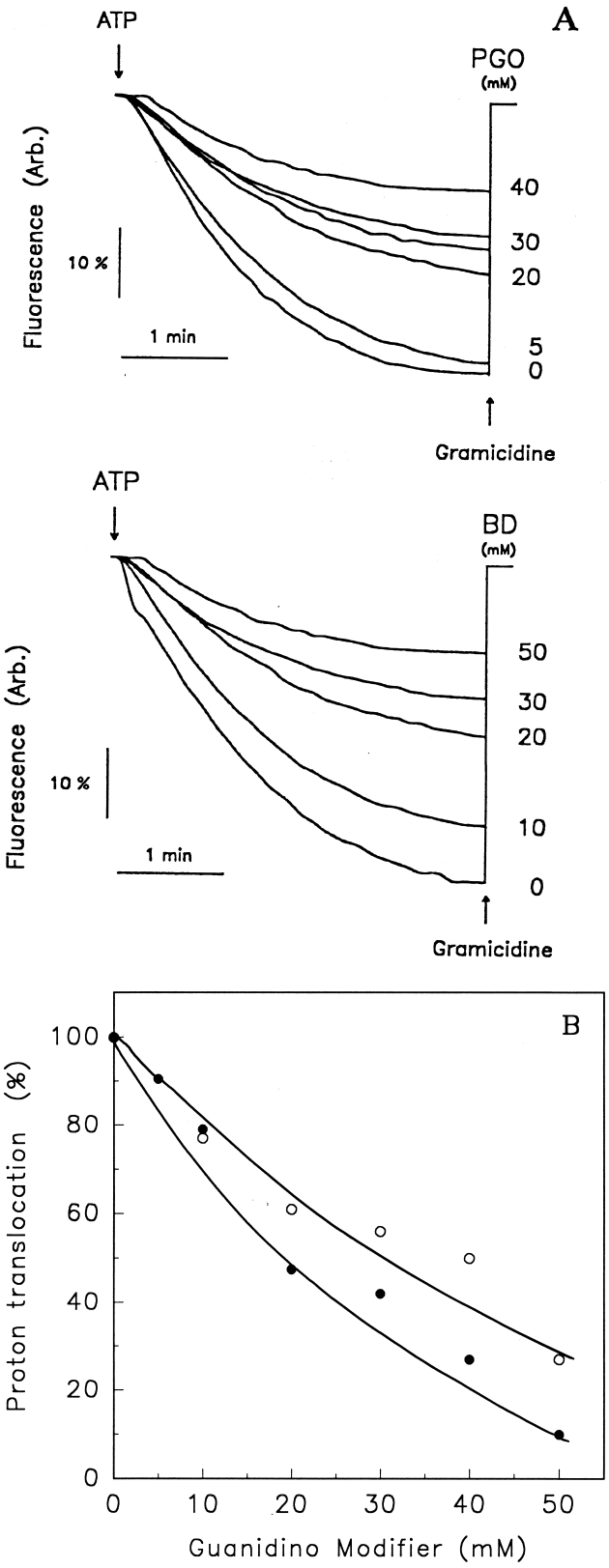


Figure 1. Inhibition of vacuolar H^+-ATPase by guanidino modifiers. Membrane protein (1.0 mg/ml) and purified ATPase (0.6 mg/ml) were incubated with various concentrations of BD (○) and PGO (●) in 50 mM Mops-KOH (pH 7) at room temperature. After 10 min, aliquots (20 µl) were removed and enzyme activity assayed as described under Materials and Methods. The control activities for membrane-bound (A) and purified ATPases (B) were 10.5 and 107.4 µmol P_i released/mg protein·h, respectively.



Semi-logarithmic time courses of BD- and PGO-inhibition of vacuolar H^+-ATPase follow a simple first order kinetics (Figure 3A and B). The first order rate constants, k_2, for BD- and PGO-inhibition of vacuolar H^+-ATPase were estimated as 0.227 and 0.416 s^{-1}, respectively (Figure 3C). Furthermore, steady-state dissociation constants, K_i, of 19.3 and 24.2 mM for BD- and PGO-inhibition of vccuolar H^+-ATPase were also calculated. The reaction order of vacuolar H^+-ATPase with BD and PGO was determined as 0.89 and 0.94 from a double-logarithmic plot of K_{obs} against BD and PGO concentrations (Figure 3D), respectively, indicating that at least one arginine residue of the enzyme was involved in the inhibition by guanidino modifiers. A Lineweaver-Burk plot of the native vacuolar H^+-ATPase yields K_m and V_{max} values of 0.39 mM and 122 μmol P_i released/mg protein·h, respectively (Figure 4). However, after incubation with guanidino modifiers, V_{max} of vacuolar H^+-ATPase remained unchanged while K_m apparently increased twofold to approximately 0.83 mM. The mode of inhibition of vacuolar H^+-ATPase by guanidino modifiers is competitive as determined by its effect on K_m but not V_{max}. It is thus confirmed kinetically that the an arginine residue is involved in the inhibition of enzymatic activity by BD and PGO and probably located at or near the catalytic site.

Protection by ATP Against BD and PGO Inhibition

To determine whether the labeling occurred at the catalytic site of vacuolar H^+-ATPase, ligand protection was employed. Table 1 summarizes protection effects by its physiological substrate ATP, inorganic phosphate, inorganic pyrophosphate, p-nitrophenol phosphate, and several nucleotides. Substantial protection of the enzyme was provided by the physiological substrate ATP in the presence of 5 mM $MgSO_4$. The addition of Mg^{2+} was crucial for the complete protection against guanidino modifiers. In its absence, the degree of protection of ligands was reduced. Other nucleotide triphosphates, which were poor substrates for ATPase, exerted widespread protection in the presence of Mg^{2+}. Protection by ATP was compared to that by the nucleotides as listed in Table 1 with and without $MgSO_4$. The order of protection of nucleotides against inhibitors was ADP > ATP > CTP > GTP > UTP > AMP. It is possible the difference in the protection effect of nucleotides might be due to the steric fitness of the ribose and diphosphate moieties of its physiological product ADP to the active pocket. Furthermore, a protective effect by phosphate and its derivatives such as pyrophosphate and p-nitrophenol phosphate was observed. Pyrophosphate provided substantial protection in the presence of Mg^{2+}. However, phosphate and p-nitrophenol

Figure 2. Inactivation of H^+-translocation by BD and PGO. Vacuolar membranes (1 mg/ml protein) were treated with various concentrations of modifiers in 50 mM Mops-KOH at room temperature for 10 min. Aliquots (20 μl) of vesicles were removed for assay of fluorescence quenching as described under Materials and Methods. The reaction was initiated by adding 3 mM $MgSO_4$. The ionophore gramicidin (2 μg/ml) was added at the end of each assay. The initial rates of fluorescence quenching were determined from the changes in fluorescence in one min. (A), Reaction trace of fluorescence quenching of Acridine Orange with various concentrations of PGO (top panel) and BD (bottom panel); (B), Concentration curves for the inhibition of H^+-translocation by modifiers. (○), BD; (●), PGO.

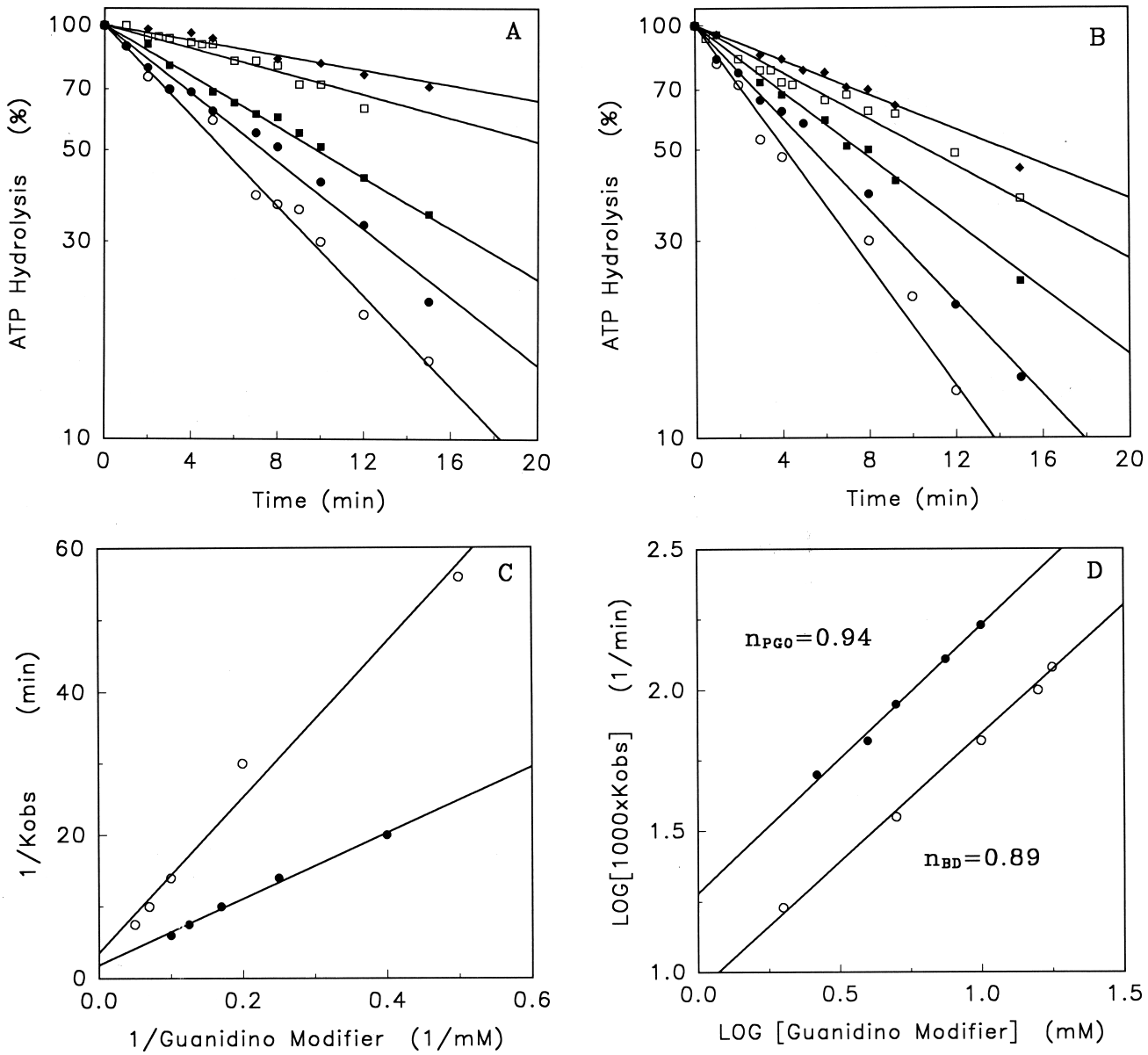


Figure 3. Kinetics of inhibition of purified vacuolar H^+-ATPase by guanidino modifiers. (A), (B) Time courses of inhibition of purified vacuolar H^+-ATPase by various concentrations of guanidino modifiers. Vacuolar H^+-ATPase (0.6 mg/ml) was incubated with various concentrations of BD and PGO in 50 mM Mops-KOH (pH 7) at room temperature. At appropriate time intervals, aliquots of solution (20 µl) were withdrawn, and the residual enzyme activity of modified vacuolar H^+-ATPase was measured as described under Materials and Methods. The BD (A) concentrations were (○) 2, (●) 5, (❍) 10, (□) 15, and (◆) 20 mM, while those for PGO (B) were (○) 2, (●), 2.5, (❍) 5, (□) 8, and (◆) 10 mM, respectively. The control activity was approximately 124.6 µmol P_i released/mg protein·h. (C) Plots of K_{obs} versus the concentration of BD and PGO. First order rate constants (k_2) and the steady-state dissociation constant of inhibitions, K_i, were each calculated directly from the slopes. (D) Double-logarithmic plots of pseudo-first order rate constants (K_{obs}) of the inhibition against concentrations of guanidino modifiers. Reaction order of 0.89 and 0.94 was determined for BD (n_{BD}) and PGO (n_{PGO}) inhibition.

phosphate exerted relatively poor protection. The results suggest that PGO and BD might modify the residue by charge-charge interaction of the guanidino group of arginine with the α and β P_i, at a site close to ribose moieties. Nevertheless, we can not exclude the possibility that PGO and BD modified the arginine residue in a domain other than the active site, inducing a conformational change and consequently the loss of enzymatic activity.

Recently, the cDNA sequence and its deduced amino acid sequence of the catalytic subunit (A) of higher plants, eubacteria, red alga, yeast, and bovine have been reported (Bowman et al., 1988; Chiu et al., 1995; Puopolo et al., 1991; Tsutsumi et al., 1991; Wilkins, 1993; Ziegler et al., 1995; Zimniak et al., 1988). The sequences of these A subunits show high homology. In addition, six regions within the sequences, designated A-F, are highly con-

Table 1. Protection of vacuolar H^+-ATPase activity against inhibition by PGO and BD.

Protectors (3 mM)	Activity as substrate (%)	Protection (%) against			
		PGO		BD	
		+Mg	-Mg	+Mg	-Mg
ATP	100.0	100.0	14.5	100.0	54.9
ADP	18.2	100.0	2.6	100.0	4.0
AMP	< 1.0	26.0	1.5	47.1	2.5
GTP	16.6	40.5	2.9	52.0	4.7
CTP	25.0	51.5	1.3	73.8	7.5
UTP	6.7	35.0	< 1.0	63.5	< 1.0
PP_i	12.3	34.7	< 1.0	37.5	< 1.0
p-nitrophenyl phosphate	2.3	10.0	< 1.0	33.8	< 1.0
P_i	–	<1.0	< 1.0	15.0	< 1.0

Vacuolar H^+-ATPase (0.6 mg/ml) was preincubated with 3 mM ligand, if present, at room temperature in the presence (+Mg) or absence (-Mg) of 5 mM $MgSO_4$. After incubation with 8 mM PGO and 10 mM BD for 10 min, aliquots (20 µl) were removed and assayed for ATPase activity. The percentage of protection was calculated according to the equation:

$$\text{Percentage protection} = \frac{[SA_{(protected)} - SA_{(unprotected)}]}{[SA_{(control)} - SA_{(unprotected)}]} \times 100$$

where SA is specific activity. The control activity of vacuolar H^+-ATPase was approximately 120–125 µmol P_i released/mg protein·h. Each value was the mean of three assays with standard deviation approximately less than 15%.

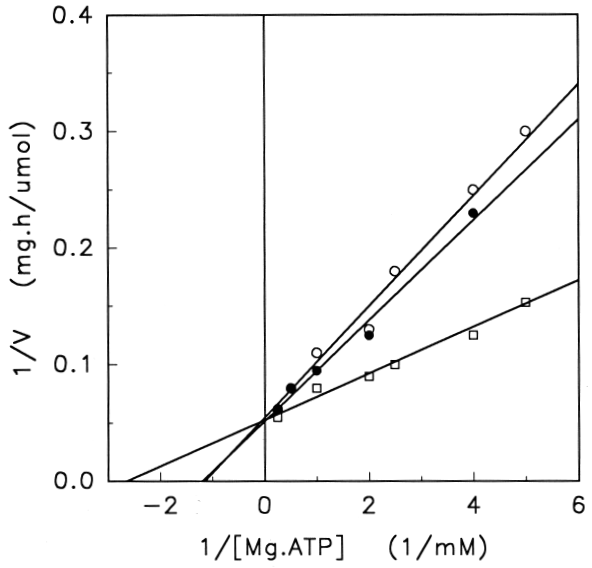


Figure 4. Lineweaver-Burk plots of BD- and PGO-inhibition of vacuolar H^+-ATPase. Purified vacuolar H^+-ATPase (0.6 mg/ml) activity was measured in the prescence of 7.5 mM BD (○), 2.5 mM PGO (●), or without modifer (□), in 50 mM Mops-KOH (pH 7) at room temperature, respectively. After 10 min, aliquots (20 µl) of enzyme were removed, and the ATPase activity was determined in an assay medium with various concentrations of ATP.

served as compared to the β (catalytic) subunit of F-type H^+-ATPases (Zimniak et al., 1988). The region B between residues 231–260 of A subunit of mung bean H^+-ATPase is homologous to residue 129–158 of the β subunit of *E. coli* F_1-ATPase, which is to contain the universal nucleotide-binding site (Nelson and Taiz, 1989). The A subunit of mung bean vacuolar H^+-ATPase contains 33 arginine residues, of which only one (Arg-234) is located in the homologous region B (Chiu et al., 1995). We speculate that Arg-234 in the region B is the most likely candidate. If this is the case, this conserved region may fold within the active pocket in the vacuolar H^+-ATPase and participate in the enzymatic activity as well as proton translocation. However, we can not exclude the possibility that modified arginine residue is located in a stretch other than the universal nucleotide binding region or that its modification may have a long range effect on the enzymatic activity. In addition, a recent report on the site-directed mutagenesis of yeast vacuolar H^+-ATPase indicates that mutation at Arg-381 of B subunit contributes to the loss of catalytic activity (Liu et al., 1996). It is also possible that the essential arginine of plant vacuolar H^+-ATPase modified by PGO and BD in this study is located at subunit B. Determinating the exact location of this essential arginine requires further investigation.

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白化綠豆幼苗液泡質子傳送核甘三磷酸水解酶中之基要精氨酸

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由白化綠豆幼苗抽出之液泡質子核甘三磷酸水解酶，經苯基乙二醛 (Phenylglyoxal) 或 2,3-丁烯雙酮 (2,3-Butanedione) 之處理，會造成核甘三磷酸水解作用與質子傳送作用的抑制現象。動力學分析得知，苯基乙二醛對液泡質子核甘三磷酸水解抑制之一級反應常數 (k_2) 與穩態解離常數 (K_i) 分別爲 0.416 s^{-1} 與 19.3 mM；而 2,3-丁烯雙酮對液泡質子核甘三磷酸水解抑制之一級反應常數 (k_2) 與穩態解離常數 (K_i) 則分別爲 0.227 s^{-1} 與 24.2 mM。同時，苯基乙二醛與丁烯雙酮對液泡質子核甘三磷酸水解抑制之級數爲 0.94 與 0.89，指證出液泡質子核甘三磷酸水解脢中至少有一精氨酸被此兩化學物所修飾。李-博氏作圖 (Lineweaver-Burk plot) 顯示此二修飾物之抑制作用爲競爭性反應。液泡質子核甘三磷酸水解酶的基質鎂$^{2+}$-核甘三磷酸可以有效的保護苯基乙二醛與 2,3-丁烯雙酮對液泡質子核甘三磷酸水解脢之抑制作用。因此，我們推論植物液泡質子核甘三磷酸水解脢反應中心具有一基要精氨酸，此一精氨酸參與基質之結合作用，並且促使苯基乙二醛與2,3-丁烯雙酮對液泡質子核甘三磷酸水解酶之抑制作用。

關鍵詞：液泡；質子傳送；核甘三磷酸水解酶；化學修飾；綠豆。