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A functional arginine residue in the vacuolar H^+-ATPase of higher plants

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The arginine-specific reagent phenylglyoxal inactivated the vacuolar H^+-ATPase of red beet. Inactivation by phenylglyoxal followed pseudo-first-order kinetics and a double log plot of the $t_{1/2}$ of inactivation versus phenylglyoxal concentration yielded a slope of 1.18. Neither inorganic anions nor DIDS protected from phenylglyoxal-mediated inactivation of the H^+-ATPase. Indeed, Cl^- stimulated the rate of phenylglyoxal-mediated H^+-ATPase inactivation relative to SO_4^{2-}. ATP, but not MgATP or ADP, protected from phenylglyoxal-mediated inactivation and inactivation resulted in a decrease in the V_{max} of the H^+-ATPase with little effect on the K_m. Collectively, these results are consistent with phenylglyoxal-mediated inactivation of the vacuolar H^+-ATPase resulting from modification of a single arginine residue in the catalytic nucleotide binding site of the vacuolar H^+-ATPase. Stimulation of phenylglyoxal-mediated inactivation by Cl^- indicates that exposure of the phenylglyoxal-sensitive functional arginine residue is enhanced in the presence of Cl^-. The failure of MgATP to protect from phenylglyoxal inactivation suggests that ATP, rather than MgATP, binds directly to the catalytic site and that Mg^{2+} may act to promote catalysis subsequent to ATP binding.

Introduction

Vacuolar-type H^+-ATPases catalyze the electrogenic transport of H^+ across the vacuolar membranes of higher plants [1], yeast [2], and fungi [3], as well as across membranes of lysosomes [4], endosomes [5], clathrin-coated vesicles [6], chromaffin granules [7], and other intracellular compartments. A common feature of these H^+-ATPases is their sensitivity to anions [8,9]. Specifically, higher plant vacuolar H^+-ATPases are stimulated by Cl^- and inhibited by NO_3^- but are insensitive to cations [10,11]. The stimulation of vacuolar H^+-ATPases by Cl^- is attributable to a dual mechanism. First, Cl^- indirectly stimulates the H^+-ATPase as a permanent anion, relieving the formation of membrane potential, thereby allowing continued H^+ transport [10]. Secondly, Cl^- interacts directly with the H^+-ATPase, stimulating its activity [10]. This second role of Cl^- in activating the H^+-ATPase can be observed in the presence of ionophores and in detergent-solubilized H^+-ATPase [12]. The sites of Cl^- permeation and Cl^- activation of the vacuolar H^+-ATPase have not been defined although evidence has been presented demonstrating that DIDS reversibly inhibits the Cl^--stimulated H^+-ATPase [13] and Cl^- permeation of the membrane [10].

Because arginine residues are positively charged at physiological pH, this amino acid has been identified as a functional residue in a number of enzymes that interact with anionic substrates [14]. The anion transport protein of red blood cells has also been shown to have an arginine residue essential for Cl^- binding and transport [15]. A class of dicarbonyl compounds has been used extensively as site-specific chemical modifying reagents to probe functional arginine residues in a number of enzymes [14]. The most widely used reagents of this class include cyclohexanedione, butanedione, and phenylglyoxal.

Because of the obvious interactions of the vacuolar H^+-ATPase with anions we have used an arginine-specific dicarbonyl reagent, phenylglyoxal, to probe the role of arginine residues in the catalytic activity of the H^+-ATPase. Our results indicate that an arginine residue is essential for H^+-ATPase catalytic activity but probably not at the site of Cl^- interaction.

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Abbreviation: DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid.

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Materials and Methods

Membrane preparation. Tonoplast-enriched membranes were prepared from the storage tissue of red beet as previously described [16]. The fraction enriched in NO_3^--sensitive H^+-ATPase (vacuolar H^+-ATPase) was collected from the 16/26% interface of a discontinuous sucrose gradient. Greater than 80% of the ATP hydrolyzing activity of this fraction was inhibited by NO_3^- and attributable to the vacuolar H^+-ATPase. The H^+-ATPase and purity of this red beet tonoplast-enriched membrane fraction has been extensively characterized [16–18].

Phenylglyoxal treatment. Following isolation, tonoplast-enriched membranes were assayed for protein [19], re-pelleted and resuspended at a concentration of 0.5 mg membrane protein/ml in 250 mM sucrose and 10 mM Tris-Mes (pH 7.0). Phenylglyoxal (Sigma) was prepared daily as a 100 mM stock in 250 mM sucrose and 10 mM Tris-Mes (pH 7.0). Appropriate volumes of phenylglyoxal stock solution or buffer were added to the membrane suspension to achieve the indicated phenylglyoxal concentration. Incubation with phenylglyoxal was carried out for the times indicated in each figure in a 30 °C water bath. Phenylglyoxal treatment was terminated by spin gel-filtration chromatography of membranes on 1 ml columns of Sephadex G-50. Each 1 ml spin column was prepared by filling a 1 ml syringe fitted with a miracloth support with Sephadex G-50 equilibrated with 250 mM sucrose, 10 mM Tris-Mes (pH 7.0). The 1 ml column was rinsed twice with buffer and twice with buffer supplemented with 0.1 mg/ml bovine serum albumin and eluted by centrifugation for 2 min at an intermediate setting in a clinical benchtop centrifuge. Following incubation with phenylglyoxal, a 100-μl aliquot of membrane suspension was applied to the spin column, and membranes collected in the void spin-through volume. Greater than 85% of the applied membrane protein was recovered and in control experiments no further inactivation by phenylglyoxal occurred following separation of membranes by spin gel-filtration chromatography. Membrane fractions were assayed for ATPase activity following phenylglyoxal treatment and membrane separation.

ATPase assay. ATPase activity was measured at 30 °C for 30 min with 5 to 20 μg protein per assay. The reaction was carried out in a volume of 0.5 ml containing 10 mM Tris-Mes (pH 7), 3 mM ATP, 3 mM $MgSO_4$ and 50 mM KCl except in Table I where KCl was omitted in some treatments. Liberated phosphate was determined by the method of Ames [20]. All assays were carried out in the absence and presence of 50 mM KNO_3 and the data reported is the NO_3^--sensitive component of ATPase activity. In control treatments greater than 80% of the total ATPase activity was NO_3^--sensitive. Assays were carried out in triplicate and individual experiments were repeated two or three times. Representative results of single experiments are shown.

Results

Inactivation of H^+-ATPase by phenylglyoxal

Incubation of the vacuolar membranes with phenylglyoxal resulted in inactivation of the H^+-ATPase (Fig. 1). Inactivation of the H^+-ATPase at concentrations between 2.5 and 10 mM phenylglyoxal followed pseudo-first order kinetics which were linear on a semi-log plot of activity versus time to a level of 95% inactivation (Fig. 1). The reaction order with respect to phenylglyoxal was estimated from a double log plot of $1000/t_{1/2}$ versus phenylglyoxal concentration (Ref. 21, see Fig. 1, inset). The slope of the plot was 1.18, suggesting that the reaction of one molecule of phenylglyoxal with each H^+-ATPase molecule is sufficient to inactivate the H^+-ATPase [21]. Thus, chemical modification of a single arginine residue results in inactivation of the H^+-ATPase.

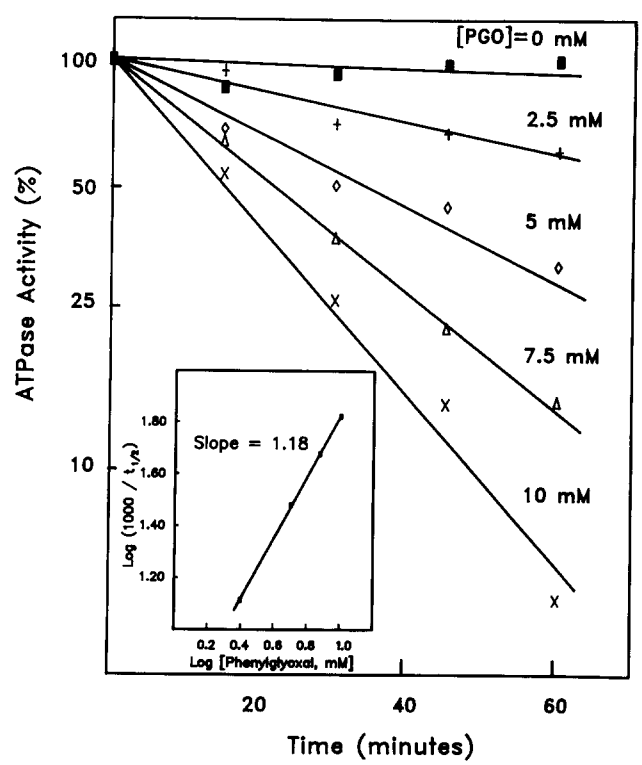

Fig. 1. Kinetics of inactivation of red beet vacuolar H^+-ATPase by phenylglyoxal. Incubation was carried out for various times with indicated concentration of phenylglyoxal (PGO) and membranes separated from phenylglyoxal by spin gel-filtration chromatography prior to assay for ATPase activity. The inset shows the double log plot of $(1000/t_{1/2})$ vs. phenylglyoxal concentration, where $t_{1/2}$ is the half-time of inactivation at each phenylglyoxal concentration. The slope of the plot gives n as 1.18, which is the number of molecules of phenylglyoxal reacting with each unit of enzyme to produce an inactive enzyme-phenylglyoxal complex [21].

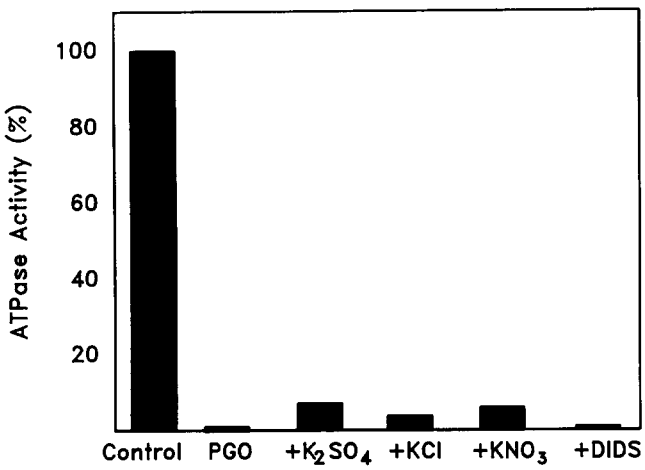

Fig. 2. Effect of inorganic anions on phenylglyoxal-mediated inactivation of red beet vacuolar H^+-ATPase. Incubation was carried out for 60 min in the absence (control) or presence of 10 mM phenylglyoxal (PGO) with the indicated salts (100 mM) or DIDS (50 μM) added 5 min prior to the incubation with PGO. Membranes were assayed for ATPase activity after separation from phenylglyoxal by spin gel-filtration chromatography.

Anion protection from phenylglyoxal inactivation

To determine if the phenylglyoxal-sensitive functional arginine residue resided in a site involved in anion stimulation of the H^+-ATPase, vacuolar membranes were incubated in the presence of phenylglyoxal and several inorganic anions (Fig. 2). None of the salts protected the H^+-ATPase from inactivation by phenylglyoxal, despite the observation that Cl^- is a potent activator and NO_3^- a potent inhibitor of the H^+-ATPase [10,11]. The anion channel blocker, DIDS, which reversibly inhibits the H^+-ATPase was also ineffective in protecting from H^+-ATPase inactivation by phenylglyoxal (Fig. 2). The time course of phenylglyoxal inactivation of the H^+-ATPase in the presence of SO_4^{2-} or Cl^- indicated that Cl^- actually accelerated the rate of phenylglyoxal inactivation (Fig. 3). These results indicated that phenylglyoxal was not acting at an arginine residing at a Cl^--protectable site, but suggested that Cl^- enhanced the exposure of the phenylglyoxal-sensitive functional arginine residue.

TABLE I

Effect of Cl^- and gramicidin on red beet vacuolar H^+-ATPase activity following partial phenylglyoxal-mediated inactivation

Incubation was carried out in the presence of 0, 3, or 5 mM phenylglyoxal for 30 min and membrane separated from phenylglyoxal by spin gel-filtration chromatography. ATPase activity was assayed as described in Materials and Methods in the absence or presence of KCl (50 mM) or in the presence of KCl (50 mM) and gramicidin (2 μM).

PGO (mM)	ATPase activity				
	μmol P_i/mg per h			% stimulation	
	−KCl	+KCl	+KCl +Gram	KCl	Gram
0	6.84	15.5	41.48	227	267
3	5.30	10.08	27.04	190	268
5	3.56	7.88	17.22	221	218

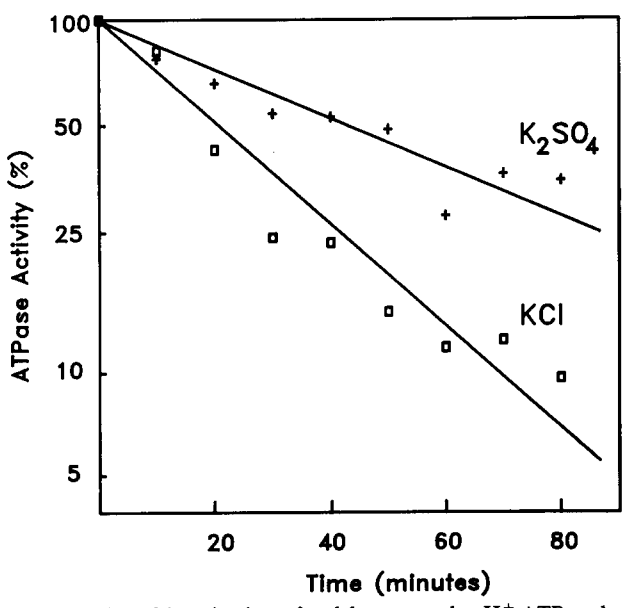

Fig. 3. Kinetics of inactivation of red beet vacuolar H^+-ATPase by phenylglyoxal in the presence of KCl or K_2SO_4. Incubation was carried out in 10 mM phenylglyoxal in the presence of either 50 mM KCl or K_2SO_4 added 5 minutes prior to incubation with PGO. Membranes were assayed for ATPase activity after separation from phenylglyoxal by spin gel-filtration chromatography.

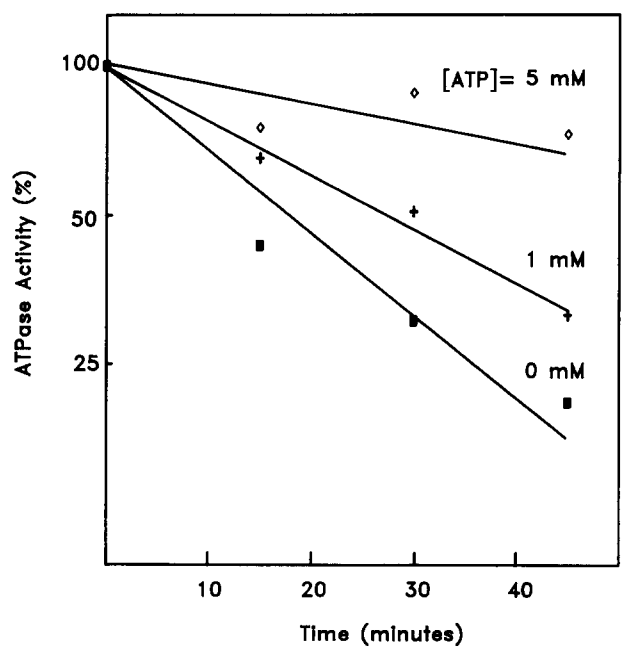

Fig. 4. Kinetics of inactivation of red beet vacuolar H^+-ATPase by phenylglyoxal in the absence or presence of ATP. Incubation was carried out in 10 mM phenylglyoxal and 0, 1, or 5 mM ATP. Membranes were assayed for ATPase activity after separation from phenylglyoxal by spin gel-filtration chromatography.

Following partial phenylglyoxal-mediated inactivation, the H^+-ATPase still demonstrated strong stimulation by Cl^- and by the proton ionophore, gramicidin (Table I). Although the basal and stimulated levels of H^+-ATPase activity were reduced following phenylglyoxal treatment, the percent stimulation by Cl^- and gramicidin was not effected, suggesting that H^+ transport or anion-stimulated functions of the H^+-ATPase were not disproportionately effected by phenylglyoxal.

Substrate protection from phenylglyoxal inactivation

Other potential anionic ligands for a functional arginine residue include the H^+-ATPase substrate, ATP. Incubation of vacuolar membranes with ATP greatly reduced the rate of phenylglyoxal-mediated inactivation (Fig. 4). Other nucleoside triphosphates and pyrophosphate were considerably less effective in protecting the H^+-ATPase from phenylglyoxal-mediated inactivation (Fig. 5). Because the substrate of the vacuolar H^+-ATPase is MgATP, we also compared the effectiveness of ATP or ADP alone or in combination with $MgSO_4$ in protecting the H^+-ATPase from phenylglyoxal-mediated inactivation. ATP alone provided the greatest protection from phenylglyoxal-mediated H^+-ATPase inactivation (Fig. 6).

Following phenylglyoxal-mediated inactivation of the H^+-ATPase, either in the absence or presence of ATP, the ATP concentration kinetics of the H^+-ATPase were determined. The effect of partial (phenylglyoxal + ATP) or complete (phenylglyoxal) inactivation was to reduce the V_{max} of the enzyme with little effect on the K_m for ATP (Fig. 7). This result indicates that phenylglyoxal reduces the number of catalytically active H^+-ATPase molecules rather than altering kinetic properties of the enzyme.

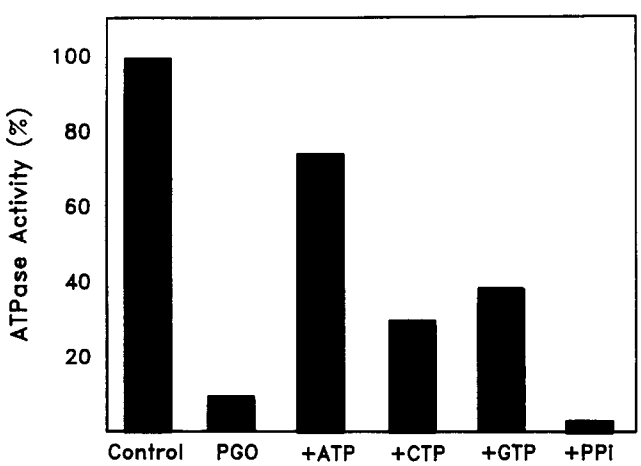

FIg. 5. Effect of several nucleoside triphosphates on phenylglyoxal-mediated inactivation of red beet vacuolar H^+-ATPase. Incubation was carried out in the absence (control) or presence of 10 mM phenylglyoxal (PGO) and the indicated nucleoside triphosphates or pyrophosphate (5 mM) for 30 min. Membranes were assayed for ATPase activity after separation from phenylglyoxal by spin gel-filtration chromatography.

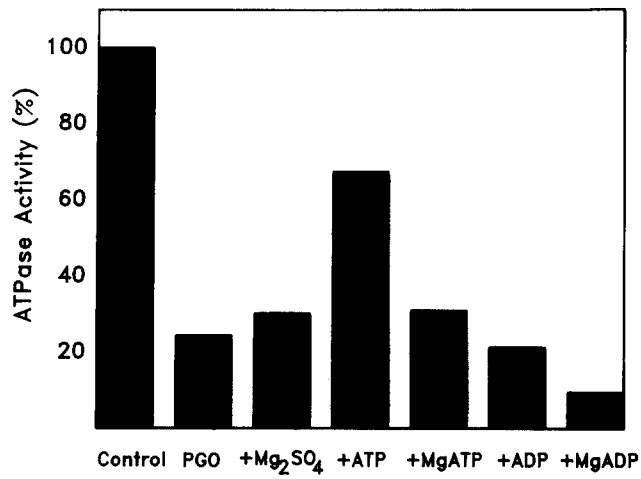

Fig. 6. Effect of Mg^{2+}, ATP, and ADP on phenylglyoxal-mediated inactivation of red beet vacuolar H^+-ATPase. Incubation was carried out in the absence (control) or presence of 10 mM phenylglyoxal (PGO) and with either $MgSO_4$ (5 mM), ATP (5 mM), $MgSO_4$ + ATP (each 5 mM), ADP (5 mM), or $MgSO_4$ + ADP (each 5 mM) for 30 min. Membranes were assayed for ATPase activity after separation from phenylglyoxal by spin gel-filtration chromatography.

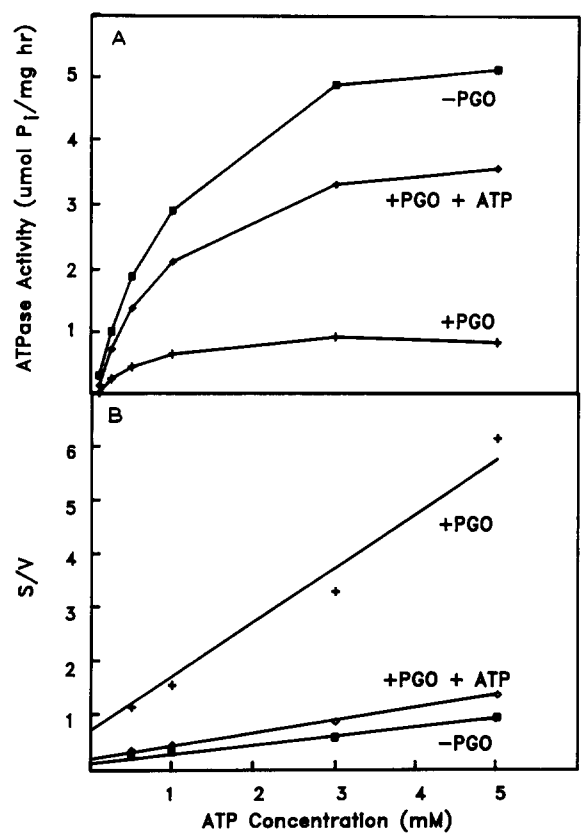

Fig. 7. ATP concentration dependence of red beet vacuolar H^+-ATPase activity before and after partial or complete phenylglyoxal-mediated inactivation. Incubation was carried out in the absence (−PGO) or presence of 10 mM phenylglyoxal without ATP (+PGO) or with 5 mM ATP (+PGO+ATP) for 30 min. Membranes were assayed for ATPase activity after separation from phenylglyoxal by spin gel-filtration chromatography. ATP and $MgSO_4$ concentrations were both varied to give the indicated concentration of both ATP and $MgSO_4$.

Discussion

Inactivation of the red beet vacuolar H^+-ATPase by phenylglyoxal suggests the presence of an essential arginine residue in this H^+-ATPase. Phenylglyoxal-mediated inactivation followed pseudo-first-order kinetics with a reaction order of approximately 1, suggesting that chemical modification of a single arginine residue results in H^+-ATPase inactivation. This result does not rule out the possibility, however, that multiple arginine residues of the H^+-ATPase that are not required for catalytic activity also react with phenylglyoxal.

The inorganic anions Cl^-, NO_3^-, or SO_4^{2-} did not protect the vacuolar H^+-ATPase from phenylglyoxal-mediated inactivation. This suggests that either the site of inorganic anion interaction with the H^+-ATPase does not possess an essential arginine residue or that such a residue is not accessible to phenylglyoxal. Interestingly, Cl^- stimulated that rate of phenylglyoxal-mediated H^+-ATPase inactivation, suggesting that Cl^- enhances exposure of the phenylglyoxal-sensitive functional arginine residue.

Phenylglyoxal-mediated inactivation of the vacuolar H^+-ATPase was partially inhibited by ATP. This suggests that the phenylglyoxal-sensitive functional arginine residue resides in a nucleotide binding site of the H^+-ATPase. Phenylglyoxal-mediated inactivation resulted in a decrease in V_{max} with little effect on the K_m for ATP. This effect of phenylglyoxal on kinetic properties of the H^+-ATPase is consistent with inactivation of an essential arginine residue in the catalytic rather than a regulatory nucleotide binding site. However, it was surprising that ATP rather than MgATP was most effective in protecting from phenylglyoxal-mediated inactivation since MgATP is the vacuolar H^+-ATPase substrate [18] and would be expected to protect from phenylglyoxal-mediated inactivation if the essential arginine is indeed present in the catalytic nucleotide binding site. A possible interpretation is that the phenylglyoxal-sensitive arginine residue is present in the catalytic nucleotide binding site and the ATP binds to a cationic site prior to Mg^{2+} binding and subsequent MgATP hydrolysis. MgATP has been shown to be effective in protecting vacuolar H^+-ATPases from inactivation by *N*-ethylmaleimide and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) [22,23], although in these previous studies the relative effectiveness of ATP and MgATP was not specifically assessed. The NBD-Cl binding domain has been proposed to comprise the catalytic nucleotide binding site of the vacuolar H^+-ATPase. Within the NBD-Cl binding domain of the 69 kDa subunit of the carrot vacuolar H^+-ATPase there is a single arginine residue that is conserved in the *Neurospora* vacuolar H^+-ATPase but not in other related H^+-ATPase subunits [24]. Also within the entire 69 kDa a subunit of the carrot H^+-ATPase there are at least six arginine residues at positions conserved between several related H^+-ATPases [24]. At the present time our data is insufficient to speculate as to which of these arginine residues may be the phenylglyoxal-sensitive site.

Acknowledgement

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References

1 Sze, H. (1985) Annu. Rev. Plant Physiol. 36, 175–208.
2 Uchida, E., Ohsumi, Y. and Anraku, Y. (1985) J. Biol. Chem. 260, 1090–1095.
3 Bowman, B.J. and Bowman, E.J. (1986) J. Membr. Biol. 94, 83–97.
4 Moriyama, Y. and Nelson, N. (1989) Biochim. Biophys. Acta 980, 241–247.
5 Mellman, I., Fuchs, R. and Helenius, A. (1986) Annu. Rev. Biochem. 55, 663–700.
6 Xie, X.-S. and Stone, D.K. (1986) J. Biol. Chem. 261, 2492–2495.
7 Cidon, S. and Nelson, N. (1986) J. Biol. Chem. 261, 9222–9227.
8 VanDyke, R.W. (1986) J. Biol. Chem. 261, 15941–15948.
9 Churchill, K.A. and Sze, H. (1984) Plant Physiol. 76, 490–497.
10 Bennett, A.B. and Spanswick, R.M. (1983) J. Membr. Biol. 71, 95–107.
11 O'Neill, S.D., Bennett, A.B. and Spanswick, R.M. (1983) Plant Physiol. 72, 837–846.
12 Bennett, A.B. and Spanswick, R.M. (1983) J. Membr. Biol. 75, 21–31.
13 Schumaker, K.S. and Sze, H. (1987) Plant Physiol. 83, 490–496.
14 Riordan, J.F. (1979) Mol. Cell. Physiol. 26, 71–92.
15 Zaki, L. and Julien, T. (1985) Biochim. Biophys. Acta 818, 325–332.
16 Bennett, A.B., O'Neill, S.D. and Spanswick, R.M. (1984) Plant Physiol. 74, 538–544.
17 Bennett, A.B. and Spanswick, R.M. (1984) Plant Physiol. 74, 545–548.
18 Bennett, A.B., O'Neill, S.D., Eilmann, M. and Spanswick, R.M. (1985) Plant Physiol. 78, 495–499.
19 Schaffner, W.C. and Weissmann, C. (1973) Anal. Biochem. 56, 502–514.
20 Ames, B.N. (1966) Meth. Enzymol. 8, 115–118.
21 Levy, H.M., Leber, P.D. and Ryan, E.M. (1963) J. Biol. Chem. 238, 3654–3659.
22 Randall, S.K. and Sze, H. (1987) J. Biol. Chem. 262, 7135–7141.
23 Bowman, E.J., Mandala, S., Taiz, L. and Bowman, B.J. (1986) Proc. Natl. Acad. Sci. USA 83, 48–52.
24 Zimniac, L., Dittrich, P., Gogarten, J.P., Kibak, H. and Taiz, L. (1988) J. Biol. Chem. 263, 9102–9112.