Plant Physiol. (1988) 87, 126–129
0032-0889/88/87/0126/04/$01.00/0

# Essential Arginyl Residues in the Plasma Membrane $H^+$-ATPase from *Vigna radiata* L. (Mung Bean) Roots[1]

Received for publication November 17, 1987 and in revised form January 18, 1988

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## ABSTRACT

**Proton-translocating ATPase ($H^+$-ATPase) was purified from mung bean (*Vigna radiata* L.) roots. Treatment of this enzyme with the arginine-specific reagent 2,3-butanedione in the presence of borate at 37°C (pH 7.0), caused a marked decrease in its activity. Under this condition, half-maximal inhibition was brought about by 20 millimolar 2,3-butanedione at 12 minutes. MgATP and MgADP, the physiological substrate and competitive inhibitor of the ATPase, respectively, provided partial protection against inactivation. Loss of activity followed pseudo-first order kinetics with respect to 2,3-butanedione concentration, and double log plots of pseudo-first order rate constants *versus* reagent concentration gave a curve with a slope of 0.984. Thus, inactivation may possibly result from reaction of one arginine residue at each active site of the enzyme. The results obtained from the present study indicate that at least one arginyl residue performs an essential function in the plasma membrane $H^+$-ATPase, probably at the catalytic site.**

The plant plasma membrane ATPase is essential to the active transport of nutrients across the plasma membrane (26). This enzyme has been purified and characterized with respect to its structural and kinetic properties (2, 5, 13, 20), its interactions with phospholipids (14), and phosphorylated reaction mechanism (4). Reconstitution studies on the purified enzyme in phospholipid vesicles have demonstrated its electrogenic $H^+$-pumping function (15, 21, 28) and provide conclusive evidence that the plant plasma membrane $H^+$-ATPase is structurally related to the $(Na^+ + K^+)$-, $Ca^{2+}$-, and $(H^+ + K^+)$-ATPases of animal cell membranes and to the $H^+$-ATPase of yeast or *Neurospora* plasma membranes. It has also been shown to differ completely from the mitochondrial type of ATPase (9) and tonoplast ATPase (17).

Recently, the gene for the yeast (25) and *Neurospora* (11) $H^+$-ATPases has been cloned and sequenced, allowing determination of the complete amino acid sequence of the polypeptide. The plant plasma membrane $H^+$-ATPase may be similar to both of these ATPases. These findings along with those on related sequences for both ATPases provide a basis for clarifying the relationship between structure and function for the plant plasma membrane $H^+$-ATPase. In particular, it seems necessary to identify the amino acid residues essential for ATP binding, hydrolysis, energy coupling, and ion translocation. However, such information in regard to the plant plasma membrane ATPase reaction mechanism is still very limited. In the case of the *Neurospora* $H^+$-ATPase, experiments with *N*-ethylmaleimide provide evidence for the presence of an essential sulhydryl group (6). Additional information can be obtained through the use of group-specific chemical probes which react covalently and cause changes in enzymic activity (22, 27).

Many reports indicate arginine residues are present in the active site(s) of enzymes that react with anionic substrates or cofactors (22, 23). This suggests the possible involvement of the positively charged guanidinium group of arginine in substrate or cofactor binding. Riordan (22) used monomeric BD[2] in borate buffer as a highly selective reagent to modify the arginine residue. In this way, an essential arginine residue was found for various ATPases, including the mammalian plasma membrane $(Na^+ + K^+)$-ATPase (7), gastric $H^+ + K^+$-ATPase (24), the sarcoplasmic reticulum $Ca^{2+}$-ATPase (19), yeast $H^+$-ATPase (8) and *Neurospora* $H^+$-ATPase (25), and the structurally distinct $F_0F_1$-type ATPases of chloroplasts (1) and mitochondria (18).

In this study, an attempt was made to determine whether arginine residues, modified by BD or a second arginine reagent, PGO, take part in ATPase reactions on plant plasma membranes. The results obtained indicate the plant plasma membrane $H^+$-ATPase possesses at least one arginine residue essential for enzymic activity.

## MATERIALS AND METHODS

**Plant Material.** Mung bean (*Vigna radiata* L.) seeds were germinated in the dark at 32°C on thin absorbent cotton on a 0.75% agar plate on an enameled tray. Following cultivation for 75 h in the dark, the root of each plant was excised and chilled in aerated cold distilled $H_2O$.

**Enzyme Preparation.** Isolation of the plasma membrane and purification of the ATPase wer conducted as previously described (13, 14). In brief, the plasma membrane was isolated from the 30 to 40% sucrose interface of an 8% dextran pellet of the microsomal fraction (10,000–80,0000*g* pellet). Solubilization of ATPase from the plasma membrane was carried out in a two-step procedure using DOC and zwittergent 3–14.

**Incubation with BD and PGO.** Modification of the ATPase with BD or PGO was conducted in MOPS-borate buffer (50 mM MOPS, 50 mM borate [pH 7.0], 0.05% asolectin) and 0 to 50 mM BD at 37°C for 0 to 30 min. $MgCl_2$ and nucleotides were included as specified in the figure lengends. Incubation was initiated by adding the ATPase to a final protein concentration of 0.4 to 0.7 mg/ml.

**ATPase Activity and Protein Determination.** ATPase activity of the modified and unmodified enzyme was measured in a standard assay mixture containing 25 mM MOPS-Mes (pH 6.5), 5 mM

[1] Support provided through special coordination funds for promoting science and technology from the Science and Technology Agency of the Japanese Government.

[2] *Abbreviations*: BD, 2,3 butanedione; MOPS, 4-morpholinepropanesulfonic acid; PGO, phenylglyoxal; DOC, deoxycholate.

$Na_2ATP$, 3 mM $MgSO_4$, 50 mM KCl, and 0.05% asolectin with 5 to 15 μg of protein in a total volume of 0.5 ml. Assays were carried out at 38°C for 5 to 30 min and terminated by adding TCA to a final concentration of 1%. Phosphate was determined by the method described previously (12). Protein was determined by the method of Bradford (3) using BSA as the standard.

**Chemicals.** BD and PGO were purchased from Sigma Chemical Co., St. Louis. The nucleotides were purchased from Boehringer Mannheim. Asolectin was purchased from Associated Concentrates Inc., New York. Stock solutions of BD and PGO were prepared just before use. All other chemicals were of analytical grade.

## RESULTS

**Comparison of Inactivation by BD and PGO.** Optimal conditions for the selective modification of the guanidinium group of arginyl residues by PGO (27) and BD (22) have already been specified. In initial experiments, the effects of BD and PGO on the activity of the plasma membrane H⁺-ATPase were examined. Incubation of H⁺-ATPase with 10 mM BD and PGO at pH 7.0 and 37°C resulted in a time-dependent exponential loss of enzymic activity. The two independent α-carbonyl reagents, PGO and BD, inactivated the H⁺-ATPase in the same manner (Fig. 1). A negative correlation between BD concentration and ATPase activity was found and noted to be essentially the same as that with PGO (Fig. 2). Thus, unless otherwise stated, BD was used as the selective modifier of arginyl residues.

**Inactivation of H⁺-ATPase by BD.** Various kinetic properties of the partially inactivated enzyme were studied (Fig. 3) after incubating the enzyme with or without 50 mM BD for 20 min. Its activity was assayed in the presence of 0 to 4.5 mM ATP for 30 min at 38°C. In the absence of BD, the $K_m$ for ATP was 1.4 mM and $V_{max}$, 2 μmol Pi/mg protein/min. In its presence, the $K_m$ was the same as that of the unmodified enzyme but the $V_{max}$ decreased to 0.3 μmol Pi/mg protein/min. The activity of the partially inactive enzyme may thus be ascribed to unmodified enzyme still present rather than to the formation of a modified enzyme having different properties.

Inactivation was dependent on BD concentration (Figs. 4 and 5). Half-maximal inhibition occurred with 50, 40, 30, 20, and 10 mM BD at about 5.1, 6.9, 9.4, 11.8, and 23.5, min, respectively (Fig. 5). As the incubation time was increased beyond 40 min, the inhibition became less linear because of irreversible inactivation of the arginine residue (Fig. 4). Inactivation kinetics were studied as a function of time and found to be pseudo-first order, according to semi-logarithmic plots of percent residual ATPase activity *versus* time (Fig. 5). The curve was log-linear up to 95% inactivation. Prolonged incubation rendered the enzyme completely inactive, while in the absence of BD, enzyme activity remained unchanged (Fig. 5). The reaction order with respect to BD in 50 mM borate buffer was determined from double-log plots of $1000/t_{1/2}$ as a function of reagent concentration, as described previously (16, 18). In such a case, a straight line should be obtained with a slope equal to $n$, the number of molecules of inhibitor reacting with each active unit of the enzyme to produce an inactive enzyme-inhibitor complex. When the data of Figure 5 were plotted in this manner, a slope of 0.984 was obtained (Fig. 5, inset), and thus inactivation appears to result from the reaction of one arginyl residue at each active site of the ATPase.

**Nucleotide Protection against BD Inactivation.** Protection against BD-induced inactivation was investigated. First, the enzyme was

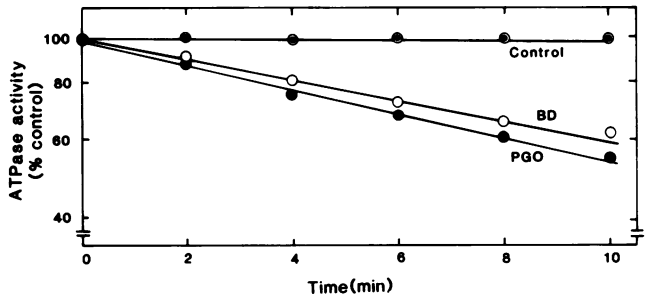


FIG. 1. Comparison of ATPase inactivation from mung bean roots by BD and PGO. The ATPase (0.5 mg/ml) was incubated at 37°C in 260 μl of 50 mM MOPS-borate buffer (pH 7.0) with 10 mM BD or PGO for 0 to 10 min. At intervals, aliquots (20 μl) were taken and assayed for ATPase activity, expressed as the percentage of the activity without BD or PGO. ATPase control activity was 1.75 μmol Pi/mg protein/min.

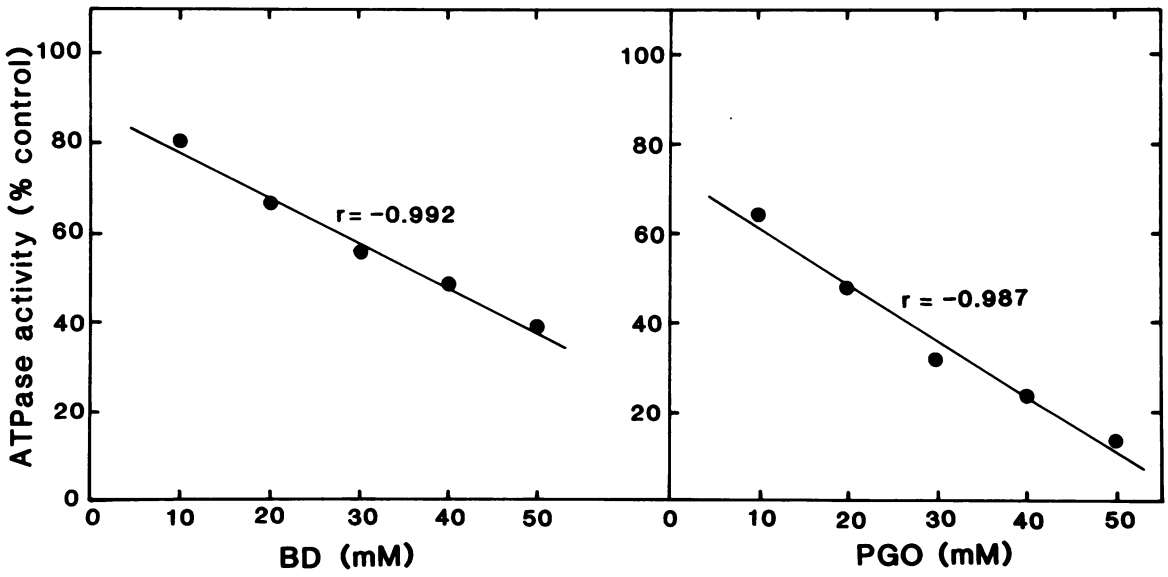


FIG. 2. Correlation between ATPase activity and BD concentration. ATPase (0.6 mg/ml) was incubated in 250 μl of 50 mM MOPS-borate buffer (pH 7.0) for 10 min at 37°C. Aliquots (20 μl) were assayed for ATPase activity, expressed as a percentage of the activity without BD or PGO. Control activity without BD or PGO was 2.07 and 1.62 μmol Pi/mg protein/min, respectively.

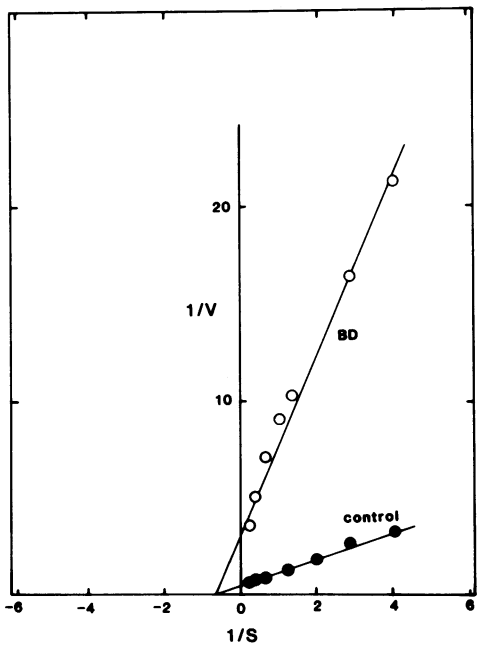


FIG. 3. Effects resulting from modification by BD on the kinetics parameters of ATPase activity. ATPase (0.4 mg/ml) was modified (○) or was not (●) by 50 mM BD in 300 μl of 50 mM MOPS-borate buffer (pH 7.0) for 20 min at 37°C. The activity of aliquots (20 μl) of these forms was measured at pH 6.5 in the assay medium containing MgATP at various concentrations.

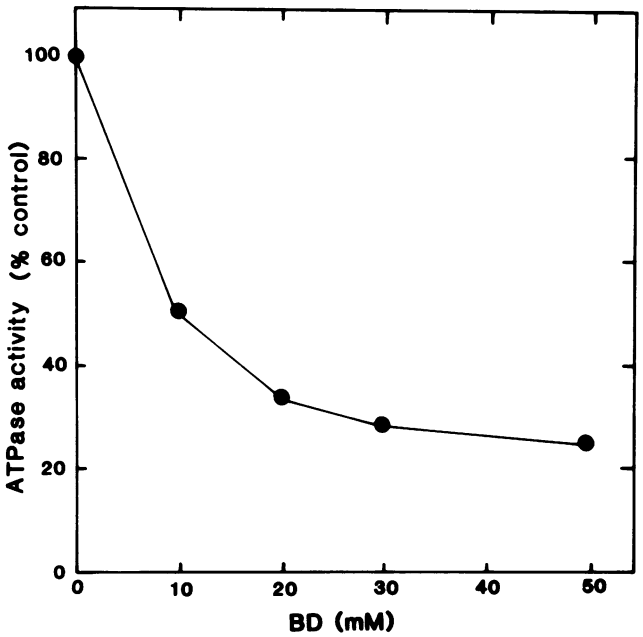


FIG. 4. Inactivation of ATPase activity as a function of BD concentration. ATPase (0.4 mg/ml) was incubated in 350 μl of 50 mM MOPS-borate buffer (pH 7.0) with 0 to 50 mM BD for 45 min at 37°C. Aliquots (30 μl) were assayed for ATPase, expressed as a percentage of the activity without BD. The control activity was 1.82 μmol Pi/mg protein/min.

incubated with 50 mM BD for 0 to 25 min in the presence of 0 to 50 mM MgADP (Fig. 6), keeping the $Mg^{2+}$ concentration constant at 10 mM. In the presence of 50 mM BD, with increasing concentration of MgADP, inactivation was prevented by as much as 80%. Table I summarizes the protective effect of nucleotides

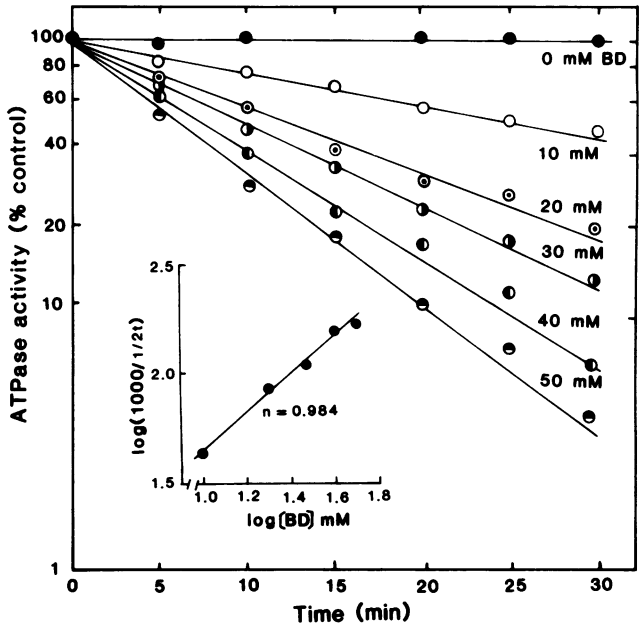


FIG. 5. Kinetics of ATPase activity inactivation by BD. ATPase (0.65 mg/ml) was incubated in 300 μl of 50 mM MOPS-buffer (pH 7.0) with 0 to 50 mM BD at 37°C for 0 to 30 min. At intervals, aliquots (20 μl) were assayed for ATPase activity, expressed as a percentage of the activity without BD. The control activity was 2.19 μmol Pi/mg protein/min. The pseudo-first order rate constant was estimated from the slope of the plots according to the equation, *ln* activity = $-kt + C$. Inset shows determined reaction order with respect to BD. Values of $t_{1/2}$ were plotted according to the equation, $\log 1000/t_{1/2} = n \log (BD) - \log K_2$, where $n$ is the reaction order.

against BD inhibition. The enzyme was incubated for 20 min with 30 mM BD in the presence of 50 mM nucleotides and protection against inactivation was noted to have the following order: ADP> ATP> GTP> *p*-nitrophenyl phosphate> AMP> UTP> CTP. CTP hardly had any effect, being hydrolyzed very poorly by the $H^+$-ATPase from mung bean roots.

## DISCUSSION

Since arginine-specific reagents of the α-dicarbonyl type such as BD and PGO (22, 27, 29) specifically modify the arginyl side chain, an increasing number of reports show arginyl residues to be essential for the action of enzymes that act on anionic substrates and cofactors (23). The present data demonstrate that two independent α-dicarbonyl reagents, BD and PGO, inactivate the plasma membrane $H^+$-ATPase of mung bean roots in much the same way. This inactivation by BD has been shown to be reversible in the absence of borate and irreversible in the presence of borate (8). BD is increasingly effective with an increase in pH from 6.0 to 8.5 (8, 25). The pH optimum for ATPase activity was 6.5 (13). Thus, in this experiment, the enzyme was treated with the inhibitor at pH 7.0 in the presence of borate. The experiments in this paper were done with 10 to 50 mM BD. This is about a 10-fold higher concentration than is used for inhibition of animal and fungal enzymes. This may be a characteristic of plant cells because the spinach chloroplast ATPase required 5 to 50 mM BD for inhibition (1). However, it cannot be ruled out that borate buffer reacts with BD to give unidentified products and to lower the effective concentration of the inhibitor available for reaction (22).

This paper is the first to report that plant plasma membrane $H^+$-ATPase activity is inhibited by incubaton with BD (Fig. 2). This inactivation is possibly due to specific modification of arginyl

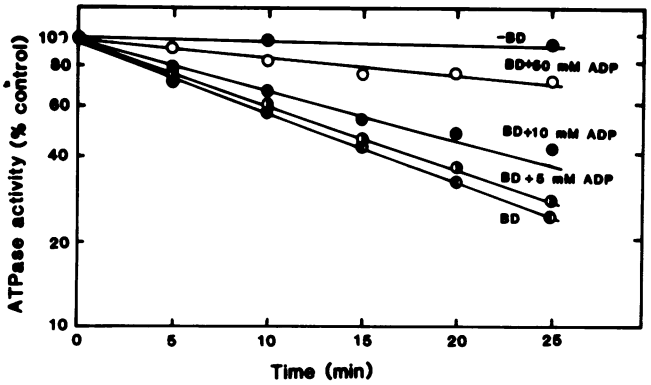


FIG. 6. MgADP protection against BD inactivation. ATPase (0.4 mg/ml) was incubated in 250 $\mu$l of 50 mM MOPS-borate buffer (pH 7.0), 50 mM BD with 0 to 50 mM MgADP at 37°C. In all cases, the free $Mg^{2+}$ concentration was maintained constant at 10 mM. At intervals, aliquots (20 $\mu$l) were assayed for ATPase activity, expressed as a percentage of the activity without BD and MgADP. The control activity was 2.3 $\mu$mol Pi/mg protein/min.

Table I. *Nucleotide Protection against Inactivation of ATPase by BD*

ATPase (0.6 mg/ml) was incubated for 20 min at 37°C in 50 $\mu$l of 50 mM MOPS-borate buffer (pH 7.0) containing 30 mM BD, nucleotides each 50 mM, and 10 mM $MgCl_2$. Aliquots (20 $\mu$l) were assayed for ATPase activity, which was expressed as a percentage of the activity without BD. Control activity without BD was 1.91 $\mu$mol Pi/mg protein/min.

| Nucleotide Added | Activity as Substrate | Inhibition |
|---|---|---|
| *50 mM* | % | |
| None | | 100 |
| ATP | 100 | 36.5 |
| ADP | 8.8 | 32.6 |
| AMP | 1.8 | 69.7 |
| GTP | 31.0 | 61.3 |
| CTP | 18.6 | 101.3 |
| UTP | 30.1 | 93.4 |
| *p*-Nitrophenyl phosphate | 4.4 | 65.4 |

residues. Kinetic data presented here indicate one essential arginyl residue to be involved at the site of inactivation (Fig. 5). MgADP, MgATP, and MgGTP partially protect BD inhibition (Table I). Protection against BD is given only by Mg nucleotides which bind to the catalytic center. Thus, there is either the presence of one arginyl residue at the substrate binding site or a general change in enzyme conformation induced by Mg nucleotides, the former possibility appearing more likely. In fact, at least one arginyl residue has been reported to have a catalytic role in the case of mammalian membrane $H^+ + K^+$-ATPase (8), $Na^+ + K^+$-ATPase (7, 10), the sarcoplasmic reticulum $Ca^{2+}$-ATPase (19), the yeast *S. prombe* $H^+$-ATPase, and the *Neurospora* $H^+$-ATPase (25).

Recently, the nucleotide sequences of the yeast (25) and *Neurospora* (11) $H^+$-ATPases, possibly similar to the plant plasma membrane $H^+$-ATPase, were determined. Kasher *et al.* (16) suggest that the essential conserved arginyl residue in *Neurospora* $H^+$-ATPase is located in the longer hydrophilic region, the exposure of which on the cytoplasmic side has been predicted (11). However, the sequence of the plant plasma membrane $H^+$-ATPase has yet to be determined.

Inclusion, the present kinetic data indicate one arginyl residue, either involved at the catalytic site or in a general change in enzyme conformation by nucleotide binding in the presence of $Mg^{2+}$, to be essential to plant plasma membrane $H^+$-ATPase activity.

## LITERATURE CITED


1. ANDREO CS, RH VALLEJOS 1977 An essential arginyl residue in the soluble chloroplast ATPase. FEBS Lett 78: 207–210
2. ANTHON GE, RM SPANSWICK 1986 Purification and properties of the $H^+$-translocating ATPase from plasma membrane of tomato roots. Plant Physiol 81: 1080–1085
3. BRADFORD MM 1976 A rapid sensitive method for the quantitative microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
4. BRISKIN DP, RT LEONARD 1982 Partial characterization of a phosphorylated intermediate associated with the plasma membrane ATPase of corn roots. Proc Natl Acad Sci USA 79: 6922–6926
5. BRISKIN DP, WR THORNLEY, JL ROTI-ROTI 1985 Target molecular size of red beet plasma membrane ATPase. Plant Physiol 78: 642–644
6. BROOKER RJ, CW SLAYMAN 1982 Inhibition of the plasma membrane ($H^+$)-ATPase of *Neurospora crassa* by N-ethylmaleimide. J Biol Chem 257: 12051–12055
7. DEPONT JJHH, BM SCHOOT, A VAN PROOYEN-VAN EEDEN, SL BONTING 1977 An essential arginine residue in the ATP-binding centre of ($N_a^+ + K^+$)-ATPase. Biochim Biophys Acta 482: 213–227
8. DIPIETRO A, A GOFFEAU 1985 Essential arginyl residues in the $H^+$-translocating ATPase of plasma membrane from the yeast *Schizosaccharomyces pombe*. Eur J Biochem 148: 35–39
9. GOFFEAU AL, CW SLAYMAN 1981 The proton-translocating ATPase of the fungal plasma membrane. Biochim Biophys Acta 639: 197–223
10. GRISHAM CM 1979 Characterization of essential arginyl residues in sheep kidney ($Na^+ + K^+$)-ATPase. Biochem Biophys Res Commun 88: 229–236
11. HAGER KM, SM MANDALA, JW DAVENPORT, DW SPEICHER, ED BENZ JR, CW SLAYMAN 1986 Amino acid sequence of the plasma membrane ATPase of *Neurospora crassa*: deduction from genomic and cDNA sequence. Proc Natl Acad Sci USA 83: 7693–7697
12. KASAMO K 1979 Characterization of membrane-bound $Mg^{2+}$-activated ATPase isolated from the lower epidermis of tobacco leaves. Plant Cell Physiol 20: 281–292
13. KASAMO K 1986 Purification and properties of the plasma membrane $H^+$-translocating adenosine triphosphatase of *Phaseolus mungo* L roots. Plant Physiol 80: 818–824
14. KASAMO K, I NOUCHI 1987 The role of phospholipids in plasma membrane ATPase activity in *Vigna radiata* L. (mung bean) roots and hypocotyls. Plant Physiol 83: 323–328
15. KASAMO K 1987 Reconstitution and characterization of $H^+$-translocating ATPase from the plasma membrane of *Phaseolus mungo* L. roots. Plant Cell Physiol 28: 19–28
16. KASHER JS, KE ALLEN, K KASAMO, CW SLAYMAN 1986 Characterization of an essential arginine residue in the plasma membrane $H^+$-ATPase of *Neurospora crassa*. J Biol Chem 261: 10808–10813
17. MANDALA, S, L TAIZ 1985 Partial purification of tonoplast ATPase from corn coleoptiles. Plant Physiol 78: 327–333
18. MARCUS F, SM SCHUSTER, HA LARDY 1976 Essential arginyl residues in mitochondrial adenosine triphosphatase. J Biol Chem 251: 1775–1780
19. MURPHY AJ 1976 Arginyl residue modification of the sarcoplasmic reticulum ATPase protein. Biochem Biophys Res Commun 70: 1048–1054
20. O'NEILL SD, RM SPANSWICK 1984 Effects of vanadate on the plasma membrane ATPase of red beet and corn. Plant Physiol 75: 586–591
21. O'NEILL SD, RM SPANSWICK 1984 Characterization of native and reconstituted plasma membrane $H^+$-ATPase from the plasma membrane of *Beta vulgaris*. J Membr Biol 79: 245–256
22. RIORDAN JF 1973 Functional arginyl residues in carboxypeptidase A. Modification with butanedione. Biochemistry 12: 3915–3923
23. RIORDAN JF, KD MCELVANY, CL BORDERS JR 1977 Arginyl residue: anion recognition sites in enzymes. Science 195: 884–886
24. SCHRIJEN JJ, WAHM LUYBEN, JJHH DEPONT, SL BONTING 1980 Studies on ($K^+ + H^+$)-ATPase. I. Essential arginyl residue in its substrate binding center. Biochim Biophys Acta 597: 331–344
25. SERRANO R, MC KIELLAND-BRANDT, GR FINK 1986 Yeast plasma membrane ATPase is essential for growth and has homology with ($Na^+ + K^+$), $K^+$- and $Ca^{2+}$-ATPases. Nature 319: 689–693
26. SZE H 1985 $H^+$-translocating ATPases: advances using membrane vesicles. Annu Rev Plant Physiol 36: 175–208
27. TAKAHASHI K 1968 The reaction of phenyl glyoxal with arginine residues in proteins. J Biol Chem 243: 6171–6179
28. VARA F, R SERRANO 1982 Partial purification and properties of the proton translocating ATPase of plant plasma membrane. J Biol Chem 257: 12826–12830
29. YANKEELOV JA JR 1972 Modification of arginine by diketones. Methods Enzymol 25: 566–579