Essential Arginy1 Residues in the Plasma Membrane H\(^+\)-ATPase from *Vigna radiata* L. (Mung Bean) Roots\(^1\)

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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 arginine 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 sulphydryl 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\(_{0}\)F\(_{1}\)-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\(_2\)O.

**Enzyme Preparation.** Isolation of the plasma membrane and purification of the ATPase were 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 micromolar fraction (10,000–80,000g 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 legends. 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

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2 Abbreviations: BD, 2,3 butanedione; MOPS, 4-morpholinepropanesulfonic acid; PGO, phenylglyoxal; DOC, deoxycholate.
Na₂ATP, 3 mM MgSO₄, 50 mM KCl, and 0.05% azoleactin with 5 to 15 μg of protein in a final 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 guanidium group of arginy1 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 arginy1 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 Kₘ for ATP was 1.4 mM and Vₘₐₓ, 2 μmol Pi/mg protein/min. In its presence, the Kₘ was the same as that of the unmodified enzyme but the Vₘₐₓ decreased to 0.3 μmol Pi/mg protein/min. The activity of the partially inactivated 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/t₁/₂ 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.](image-url)

![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.](image-url)
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 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 \(\alpha\)-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 \(\alpha\)-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 incubation with BD (Fig. 2). This inactivation is possibly due to specific modification of arginyl

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 30 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.

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 t1/2 were plotted according to the equation, log 1000/t1/2 = n log (BD) – log K2, where n is the reaction order.
residues. Kinetic data presented here indicate one essential arginy1 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 arginy1 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 arginy1 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. Kasamo et al. (16) suggest that the essential conserved arginy1 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 arginy1 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**

25. Serrano R, MC Kielland-Brandt, GR Finn 1986 Yeast plasma membrane ATPase is essential for growth and has homology with (Na\(^+\) + K\(^+\)) - and Ca\(^2+\)-ATPases. Nature 319: 689–693

**Fig. 6.** MgADP protection against BD inactivation. ATPase (0.4 mg/ml) was incubated in 250 μl 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 μl) were assayed for ATPase activity, expressed as a percentage of the activity without BD and MgADP. The control activity was 2.3 μmol 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 μl of 50 mM MOPS-borate buffer (pH 7.0) containing 30 mM BD, nucleotides each 50 mM, and 10 mM MgCl\(_2\). Aliquots (20 μl) were assayed for ATPase activity, which was expressed as a percentage of the activity without BD. Control activity without BD was 1.91 μmol Pi/mg protein/min.

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<th>Nucleotide Added</th>
<th>Activity as Substrate</th>
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