

**ANTIMYCIN A INHIBITION OF PROTON PUMPING
ASSOCIATED WITH ROOT PLASMA MEMBRANE H^+ -ATPASE**

ABSTRACT

Plasma membrane vesicles obtained by discontinuous sucrose gradient centrifugation from corn roots, exhibited vanadate-sensitive H^+ -ATPase activity with minimum contaminations of other subcellular ATPases and phosphatase activities. The H^+ -ATPase catalyzed the hydrolysis of Mg-ATP and supported a proton pumping process. The presence of antimycin A had no significant effects on the rate of catalyzed ATP hydrolysis. However, proton pumping activity was substantially reduced. Time courses of proton pumping were analyzed by a kinetic model which allowed an accurate determination of the initial pumping rate (R_0) and the proton leakage of the energized membrane (k_1) from steady-state properties. With a rapid quenching of ATP hydrolysis by the use of hexokinase, the proton leakage of de-energized membrane (k_2) was also determined. This analysis indicated that antimycin A inhibition was characterized by a decrease in R_0 without any significant effects on both k_1 and k_2 . Possible implications of this differential inhibition on coupled ATPase activities are discussed.

INTRODUCTION

The plasma membrane of plant root cells is known to contain a vanadate-sensitive H^+ -ATPase (Serrano 1989) which is characterized by the involvement of a

phosphorylated intermediate in the catalytic pathway. The enzyme utilizes the energy released from ATP hydrolysis to drive a proton pumping process that leads to the establishment of a proton electro-chemical potential ($\Delta\mu_{H^+}$) across the membrane (Briskin and Hanson 1992). It is believed that this $\Delta\mu_{H^+}$ is the driving force for the uptake of soil nutrients by plants (Briskin 1990; Michelet and Boutry 1995).

The molecular weight of this monomeric enzyme is about 100,000 kDa (Briskin and Leonard 1982). Based on primary amino acid sequence, it has been predicted that the protein may contain up to 10 transmembrane segments with the catalytic site for ATP hydrolysis located at the protein domain exposed to cell cytoplasm (Wach *et al.* 1992). The enzyme may transport up to one proton per ATP molecule hydrolyzed (Brauer *et al.* 1989; Briskin 1990). It has been shown (Briskin 1986) that the proton pumping process probably occurs after the formation of the phosphorylated intermediate. However, the exact molecular mechanism by which ATP hydrolysis pathway is coupled to the proton pumping process remains elusive (Briskin and Hanson 1992).

In principle, the coupling may be either direct or indirect. The direct coupling is characterized by sharing at least one common molecular event to link the two processes together. This arrangement is exemplified by the "Q-cycle" type arrangement of oxidative phosphorylation in which the redox reaction is directly responsible for the obligatory transfer of both electrons and protons (Mitchell 1976). Events which could link phosphoryl group transfer and proton pumping, are yet to be identified for plant root plasma membrane H^+ -ATPase. Nevertheless, direct mechanism predicts a rigid interdependence between coupled events. Thus, external perturbations would affect coupled activities to the same extent, both qualitatively and quantitatively. The coupling may also be indirect, in the sense that long range conformational intermediate interaction step(s) are needed to link separated pathways for ATP hydrolysis and proton pumping (Boyer 1988). The intermediate steps may function as switches or valves to transfer energy released from ATP hydrolysis to activate proton pumping. This indirect arrangement would allow differential responses of coupled events to external influences (Ernster 1977). Thus, as previously described (Briskin 1990), it is possible to qualitatively define the nature of the coupling of the plasma membrane H^+ -ATPase based on the responses of the two activities to imposed conditions (Tu *et al.* 1994).

In this work, we tested the effects of antimycin A on the coupled activities of the vanadate-sensitive H^+ -ATPase in corn-root plasma membrane vesicles obtained through discontinuous gradient centrifugation. We found that the antibiotic inhibited the proton pumping, but had no significant effect on the hydrolysis of ATP. These results suggest that an indirect coupling mechanism may be operational.

MATERIALS AND METHODS

Isolation of Plasma Membrane

Microsomal fractions from corn roots were isolated as previously described (Brauer *et al.* 1992). Briefly, corn seeds (*Zea mays* L. Cv. WF7551, Custom Farm Seeds Co.) were germinated on filter paper moistened with 0.1 mM CaCl₂ for 3 days at 28C. Approximately 60 g of excised roots were homogenized with a motor and pestle at 4C in the isolation medium containing 0.3 M sucrose, 5 mM ethyleneglycol-bis(β -aminoethylether-N,N'-tetraacetic acid) (EGTA), 5 mM β -mercaptoethanol, 5 mM dithiothreitol (DTT), and 0.1 M Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.7 (adjusted at 4C). After filtration through four layers of cheesecloth, the homogenate was subjected to differential centrifugation at 6,000 g for 20 min and 90,000 g for 40 min, to obtain microsomal pellets. The pellets were suspended in isolation medium and centrifuged again at 90,000 g for 40 min. The washed microsomes were suspended in the isolation medium (2 mg protein mL⁻¹) and layered over a discontinuous density gradient consisting of 34 and 42% (w/w) sucrose buffered with 5 mM Hepes, pH 7.5. After centrifugation at 100,000 \times g for 150 min, the membrane vesicles collecting between 35 and 42% sucrose steps were removed, diluted 3- to 4-fold with 5 mM Hepes (pH 7.5) and then centrifuged at 100,000 \times g for 90 min. The final pellets were suspended in 20 mM Hepes (pH 7.5), 0.2 M sucrose, and 10% (w/v) glycerol at 5 mg protein mL⁻¹.

Measurement of Coupled ATPase Activities

About 100 μ g of purified plasma membrane vesicles were diluted with 2.0 mL of assay medium containing 20 mM Mes titrated to pH 6.45 with BTP, 2.5 mM MgSO₄, 1 mM EGTA, 0.1 mM molybdate, 50 mM KNO₃, 7.5 μ M acridine orange (AO), 5 mM glucose, various concentrations of antimycin A with or without 0.2 mM vanadate, and incubated at 22C for 10 min. The ATPase reactions were then initiated by the addition of 20 μ L of 0.1 M ATP titrated to 6.45 with BTP. Specific procedures for measurement of ATP hydrolysis and proton pumping are as follows:

ATP Hydrolysis. After initiation, ATP hydrolysis was allowed to continue for 10 min at 22C before termination by addition of 1 mL of ice cold 5% trichloroacetic acid. ATP hydrolysis catalyzed by the H⁺-ATPase was assayed by the direct measurement of inorganic phosphate released using the malachite green-molybdate assay (Tu *et al.* 1987). Plasma membrane H⁺-ATPase activity was

expressed as the difference between assays with and without vanadate. Vanadate-sensitive activity normally accounted for about 70% of the total ATP hydrolysis associated with the plasma membrane vesicles. Furthermore, the rate of vanadate-sensitive ATP hydrolysis remained constant for about 15 to 20 min under the experimental conditions used. Thus, the average rate over a period of 10 min was used to represent the initial hydrolysis rate (R_{ATP}) of the H^+ -ATPase.

Proton Pumping and Membrane Proton Leakage. After initiation, ATP supported H^+ -transport was followed by changes in the absorbance of acridin orange at 492.5 nm using a Beckman DU-70 Spectrophotometer interfaced to a personal computer. The digitized data (sampling time = 1 s) were then analyzed according to a steady-state kinetic model developed in this laboratory (Tu *et al.* 1987). The model assumed that gradient formation also induced a membrane proton leakage and thus, gradually decreased the net proton transport. When the net transport rate approached zero, i.e. when leakage equaled to initial pumping rate, a steady-state condition was reached. Mathematically, ATP-supported proton pumping was represented by the following equations:

$$\text{net proton pumping rate} \quad d\delta/dt = R_o - k_1\delta \quad (1)$$

$$\text{steady-state approximation} \quad R_o = k_1\delta_s \quad (2)$$

$$\text{time-course of pumping} \quad \ln(1 - \delta/\delta_s) = -k_1t \quad (3)$$

where δ , δ_s , R_o and k_1 represent the extent of proton transport, the extent of transport at steady state, initial proton pumping rates, and pumping inhibition constants (proton leakage with ATPase activity on), respectively. Initial ATP hydrolysis rates (R_{ATP}) and initial pumping rates (R_o) were related:

$$R_{ATP} = mR_o \quad (4)$$

where "m" represents the stoichiometric ratio or the extent of coupling between proton pumping and ATP hydrolysis. A decrease in the value of "m" should reflect a decrease in utilization of ATP hydrolysis energy to support proton pumping, resulting in a decrease in net proton uptake by the plasma membrane vesicles.

A rapid quenching of ATP hydrolysis could discharge the proton gradient δ_s by leakage through the de-energized membranes. Hexokinase-induced phosphorylation of glucose was utilized to rapidly exhaust added ATP. Proton leakage follows a simple first-order decay:

$$\ln(\delta/\delta_s) = -k_2t \quad (5)$$

where δ represents the residual gradient at time t after the quenching. It should be noted that k_2 represents the proton leakage constant of de-energized (ATP hydrolysis silent) membranes.

Protein Assay

Protein concentrations were determined by a modified Lowry procedure after precipitation by TCA in the presence of deoxycholate (Bensadoun and Weinstein 1976).

Materials

Actimycin A, ATP (Na-salt), DTT, EGTA, sucrose and all buffers were obtained from Sigma.

RESULTS

Membrane Purity

Plasma membrane vesicles purified by sucrose density-gradient centrifugation, normally accounted for 90% of the total vanadate-sensitive ATPase activity (Tu *et al.* 1994). The residual contamination originated from tonoplast ATPase and nonspecific phosphatase which could be inhibited by bafilomycin (or nitrate) and molybdate, respectively. The presence of oligomycin, a mitochondrial ATPase inhibitor, had a negligible effects on the total ATPase activity measured. These results suggested that the plasma membrane preparation was largely free from contamination by the mitochondrial inner membrane. This observation agreed with our previous finding (Tu *et al.* 1994) that the membrane preparation contained insignificant amounts of antimycin-A sensitive, mitochondrial electron transfer activity (from NADH to oxygen). Mitochondria-free plasma membrane vesicles exhibited ATP supported proton pumping which could be terminated by a rapid exhaustion of ATP (Fig. 1, trace A) using glucose and hexokinase.

Antimycin A is a powerful and specific inhibitor of the mitochondrial electron transfer between cytochromes b and c_1 . For example, in isolated rat liver mitochondria, the electron transfer may be completely inhibited by $\sim 0.5 \mu\text{M}$ antimycin A (Lam and Tu 1980). To our knowledge, antimycin A has never been evaluated as an inhibitor of any known plasma membrane ATPases. However, we noted that ATP supported proton uptake in the plasma membrane vesicles was significantly inhibited by the presence of antimycin A (Fig. 1, trace B). The apparent decline in the pumping rate, in addition to a direct interaction with the

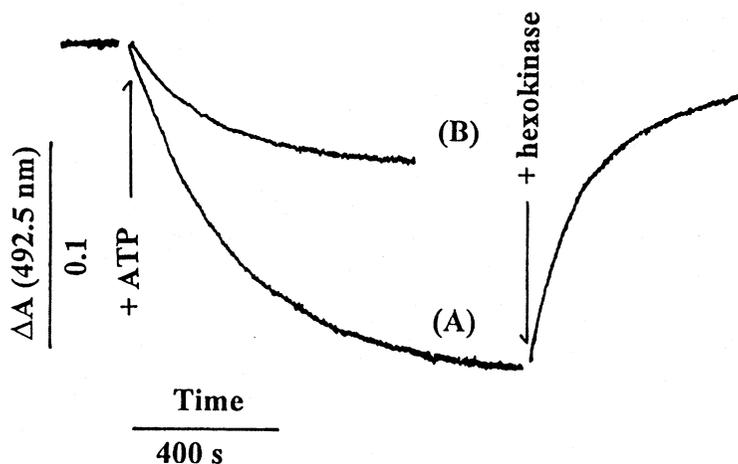


FIG. 1. EFFECTS OF ANTIMYCIN A ON THE TIME COURSE OF ATP-SUPPORTED PROTON UPTAKE

Membrane vesicles (85 μg) were incubated in proton pumping medium with or without antimycin A as described in text. Trace (A) showed a typical time course of proton pumping as measured by the absorbance change of acridine orange at 492.5 nm. Pumping was initiated by the addition of ATP and terminated by the addition of hexokinase (30 units added, 1 unit is defined as phosphorylation of 1 μmole of glucose per min). The amounts of hexokinase added could exhaust all ATP (1 μmole) in about 2 s. The proton pumping time course of the vesicles, after incubation with 28 μM of antimycin A, is represented in trace (B).

pumping mechanism, could also be attributed to several other causes including inhibition of ATP hydrolysis, enhanced membrane proton leakage, etc. Further experiments were therefore performed to identify the origin of observed inhibition.

Effects of Antimycin A on ATPase Activity

We first tested the response of the ATPase activities to different concentrations of antimycin A. As shown (Fig. 2, trace A), the rate of ATP hydrolysis (R_{ATP}) of the vanadate-sensitive ATPase was practically unaffected by antimycin A in the tested concentration range. In contrast, antimycin A exhibited a strong inhibition (Fig. 2, trace B) to the proton pumping rate (R_0). Nearly a 50% reduction in the pumping rate was observed with about 14 μM of antimycin A. With levels of antimycin A approaching 28 μM , the pumping rate was further reduced to $\sim 60\%$. From the kinetic equation (4) that delineated the relationship between

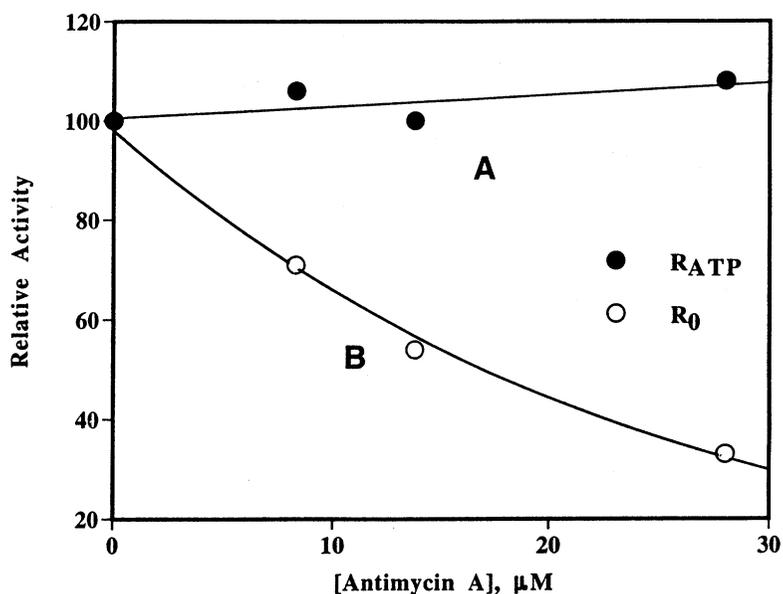


FIG. 2. DIFFERENTIAL EFFECTS OF ANTIMYCIN A ON COUPLED ATPASE ACTIVITIES

Approximately 95 μg of plasma membrane vesicles were incubated in proton pumping medium containing indicated concentrations of antimycin A for 10 min at 22C before the addition of ATP. The initial rates of ATP hydrolysis and proton pumping of the ATPase were determined as described in text. The rates (506.3 $\text{nmol P}_i \text{ released min}^{-1} \text{ mg}^{-1}$ for R_{ATP} and 0.2750 $\Delta A \text{ min}^{-1} \text{ mg}^{-1}$ for R_0) obtained in the absence of antimycin A were assigned as 100%.

R_0 and R_{ATP} , the kinetic consequence of antimycin A to the coupled ATPase activities could be assigned to a decrease in the value of "m" (Table 1). This result suggested that the mode of action of antimycin A is to decouple the proton pumping process from ATP hydrolysis.

Nonprotonophore Effects of Antimycin A

Certain weak organic acids, such as 2,4-dinitro phenol, carbonyl cyanide *m*-chlorophenylhydrazone, etc., may discharge the proton gradient by rapidly shuttling protons across the membrane (McLaughlin 1981). This equilibrium of proton concentration also leads to an acceleration of ATP hydrolysis since the two processes are coupled. Antimycin A, with the following chemical structure, is not generally considered as a protonophore.

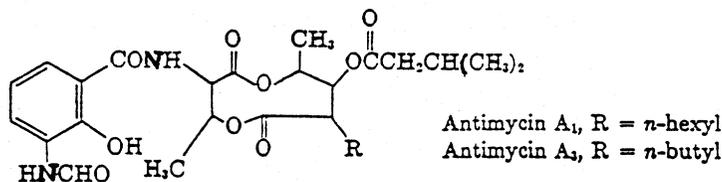


TABLE 1.
EFFECTS OF ANTIMYCIN A ON THE RATES OF ATPASE ACTIVITIES¹.

Antimycin A added (μM)	R_{ATP} ($\text{nmol P}_i \text{ mg}^{-1} \text{ min}^{-1}$)	R_0 ($\Delta\text{A mg}^{-1} \text{ min}^{-1}$)	"m" (R_0/R_{ATP}) $\times 10^4$
0	485.2	0.2680	5.52
8.4	514.4	0.1916	3.73
14	485.2	0.1432	2.95
28	524.0	0.0872	1.66

¹ ATPase activities were measured as described in Materials and Methods. The data shown represent the average of two independent runs with a maximum error as $\pm 10\%$. Activities R_{ATP} and R_0 are expressed per mg of plasma membrane protein.

The data shown in Table 1 demonstrate that antimycin A did not significantly affect ATP hydrolysis rates. Thus, it is unlikely that antimycin A functions as a protonophore in the plasma membrane vesicles. Furthermore, antimycin A had no apparent effects on proton leakage, measured as k_1 , of the energized membrane (Table 2). We also tested the effect of antimycin A on the proton leakage of de-energized membranes (k_2). Experimentally, ATP supported proton pumping in the presence of different concentrations of antimycin A, was allowed to reach a steady state. Excess amounts of hexokinase were then added in to rapidly deplete all of the ATP (in less than 2 s) by phosphorylating glucose present. Without continual ATP hydrolysis to sustain pumping, the accumulated proton would leak back and return to the initial state (Fig. 1, trace A). We found (Table 2) that antimycin A did not affect the value of k_2 . Thus, the insensitivity of R_{ATP} , k_1 , and k_2 to antimycin A indicate that antimycin A does not function as a protonophore.

TABLE 2.
EFFECTS OF ANTIMYCIN A ON MEMBRANE PROTON LEAKAGE ¹.

Antimycin A (μM)	k_1 (min^{-1})	k_2 (min^{-1})
0	0.304	0.192
14	0.320	0.181
28	0.314	0.217

1. Membrane leakage rate constants were determined according to Equations (3) and (5) from digitized time courses of proton movement. The data shown represent averages of two independent experiments with a maximum error of $\pm 10\%$.

DISCUSSION

Antimycin A is a classical inhibitor of the activity of the electron transfer chain in the mitochondrial inner membrane. To our knowledge, this compound has never been implicated in any known membrane proton pumping processes. The current investigation demonstrated that antimycin A interfered with proton pumping process associated with the vanadate-sensitive H^+ -ATPase of corn root plasma membrane vesicles. Kinetic analysis showed that antimycin A slowed down the H^+ pumping rate without significant effect on the rate of ATP hydrolysis. These results, together with the fact that antimycin A did not change membrane proton leakage under different energetic conditions, suggest a decoupling of proton pumping from ATP hydrolysis. The observed effects of antimycin A are different from those of weak organic acid-type of protonophores which discharge proton gradient by rapidly shuttling protons across membranes. However, the molecular origin of these effects is unknown. Whether antimycin A exerts similar effects to other types of membrane H^+ -ATPases also remains to be established. The observation that two coupled events, ATP hydrolysis and H^+ -pumping, exhibited differential responses to antimycin A, supported an indirect-linkage between the two events as proposed in previous reports (Briskin and Hanson 1990; Tu *et al.* 1994).

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