

ALUMINUM INHIBITION OF NADH-LINKED ELECTRON  
TRANSFER BY CORN ROOT PLASMA MEMBRANES

Matthew Loper, David Brauer, Deidre Patterson, and Shu-I Tu

**ABSTRACT:** Vesicles enriched in right-side-out plasma membranes were isolated from corn roots by a modified two-phase partition method. Using reduced nicotinamide adenine dinucleotide as electron donor, isolated vesicles were capable of reducing both ferricyanide and oxygen. At pH 6.0, the presence of Al inhibited both reduction processes to a similar extent. This result indicates that these two reduction processes share at least one common step that is sensitive to Al. Inhibition was not associated with a change in the structure of the membrane domain, as revealed by fluorescence polarization of membrane incorporated probes. These results are the first indications that the electron transfer processes of plasma membranes are sensitive to the presence of Al.

INTRODUCTION

Aluminum toxicity of plant roots has been an area of intensive study (1). To date, the primary lesion for Al remains unknown. Some of the phenomena associated with Al toxicity include: inhibition of ion uptake (2), disruption of cellular regulation by calmodulin (3), and inhibition of mitosis in root apices (4). Many of these effects induced by Al result from the interaction between Al and phosphate groups (1), and Al and other multivalent cations like Ca (3). Enzymes that utilize substrates with a terminal phosphate group are extremely sensitive to inhibition by Al. For example, the activity of glucose-6-phosphate dehydrogenase,

which catalyzes the reduction of the substrate to ribulose-5-phosphate, is greatly reduced in the presence of Al ions (5).

Membranes also would be considered a likely target for Al inhibition because of the relatively high abundance of phosphate groups from phospholipids. Changes in the structure of isolated membranes have been detected when assayed in the presence of Al (6). The consequences of these changes in membrane structure on the enzymatic functions of membranes has not been studied in detail. The effects of Al have been studied on the activity of the plasma membrane-bound H<sup>+</sup>-translocating ATPase (7). Inhibition of enzyme activity occurs at Al concentrations sufficiently low as not to alter membrane structure as assayed by changes in the polarization of the membrane incorporated probe diphenyl-hexatriene (DPH). Kinetic characterization reveals that Al inhibition of the H<sup>+</sup>-translocating ATPase probably interferes with the transfer of the phosphate from the substrate Mg-ATP to the enzyme to form a catalytic intermediate. Therefore, studies concerning the Al inhibition of the H<sup>+</sup>-translocating ATPase can not provide definite results concerning the consequences of Al on membrane structure and associated enzymatic activities because of the probable effects of Al on phosphate transfer. In study, we choose to study the Al inhibition of reduced nicotinamide adenine dinucleotide (NADH) linked electron transfer by purified right-side out (RSO) plasma membranes from corn roots. This system was chosen because this membrane leaflet would be more likely to be exposed to Al and the reaction mechanism of this enzyme system does not involve the transfer of a phosphate moiety.

## MATERIALS AND METHODS

**Membrane Isolation.** Corn seeds (*Zea mays* L. cv. WF9 x Mo17) were germinated on filter paper saturated with 0.1 mM CaCl<sub>2</sub> in the dark at 28 C for 3 days as described previously (8). Excised roots were homogenized in grinding buffer containing 25 mM bis-tris-propane (BTP) titrated to pH 7.8 with 2-[N-Morpholino]ethanesulfonic acid (MES), 0.25 M sucrose, 10 % (w/v) glycerol, 2 mM ethylene glycol-bis(B-aminoethyl ether) N, N, N', N'-tetraacetic acid, 2 mM MgSO<sub>4</sub>, 2 mM ATP, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 % (w/v) bovine albumin serum and 5 mM dithiothreitol. The supernatant resulting from a 6,000g spin for 20 min was centrifuged at 90,000g for 40 min to obtain a microsomal pellet. The microsomal pellet was suspended in 0.25 M sucrose, 4 mM KCl and 4 mM K-phosphate (pH 7.8). RSO plasma membranes were purified from the

microsomes by aqueous polymer 2-phase partitioning according to a protocol similar to that of Widell and Larsson (9). Two g of resuspended microsomes were added to 8 g of two-phase system containing final concentrations of 6.3 % (w/v) polyethylene glycol (average molecular weight of 3,350 daltons) and 6.3 % (w/v) dextran T-500. After three partitioning, the upper phases were combined and diluted three-fold with resuspension buffer containing 2 mM BTP titrated to pH 7.2 with MES, 0.25 M sucrose and 10 % (w/v) glycerol. Membranes enriched in RSO plasma membranes were collected by centrifugation and resuspended in resuspension buffer.

Purification of RSO plasma membranes were followed by increases in the activity of vanadate-sensitive ATP hydrolysis stimulated by 0.02 % (w/v) Triton X-100 (10). Two phase partitioning increased the specific activity of Triton stimulated, vanadate sensitive ATP hydrolysis by over two-fold from 3.4  $\mu\text{mol Pi released/ mg protein/ h}$  in microsomes to 8.2  $\mu\text{mol Pi released/ mg protein/ h}$  in the final pellet. These preparations of RSO-plasma membranes were essentially devoid of endoplasmic reticulum, golgi and mitochondria based on the lack of the activities of cytochrome c reductase, latent IDPase and cytochrome c oxidase, respectively, as assayed by the procedures mentioned previously (11). These results indicated that RSO plasma membranes purified by the described protocol were of high purity, similar to that reported previously for other tissues (9, 10,12).

**Electron Transfer and ATP Hydrolysis Assays.** Oxygen consumption was measured with a YSI model 53 oxygen monitor using a buffer containing 0.25 M sucrose, 10 % (w/v) glycerol and 20 mM MES titrated to pH 6.0 with BTP. After attaining a basal rate of oxygen consumption in the presence of vesicles, NADH was added to a concentration of 1.5 mM. Electron transfer also was determined by monitoring the reduction of ferricyanide at 420 nm. The hydrolysis of MgATP was determined in the presence of 50 mM  $\text{KNO}_3$  and 0.02 % (w/v) Triton X-100 by following the release of inorganic phosphate as described previously (13). The difference in ATP hydrolysis obtained in the absence and presence of 0.2 mM vanadate was used to represent the vanadate-sensitive ATPase activity of the plasma membrane (13). Each experiment contained at least 3 replicates and each experiment was performed at least twice. The coefficient of variation for each mean was less than 5 % and did not exceed the size of the data symbol.

**Evaluation of Membrane Structure.** Changes in membrane structure can be evaluated by changes in the polarization of fluorescence of lipophilic probes (14).

RSO plasma membranes were incorporated with either DPH or the DPH analog of PC (DPH-PC) as described previously (15). Polarization of the incorporated probes was determined after measuring the fluorescence intensities at 424 nm with vertically polarized excitation light at 370 nm by calculating the ratio of parallel intensity minus perpendicular intensity divided by the sum of the two intensities.

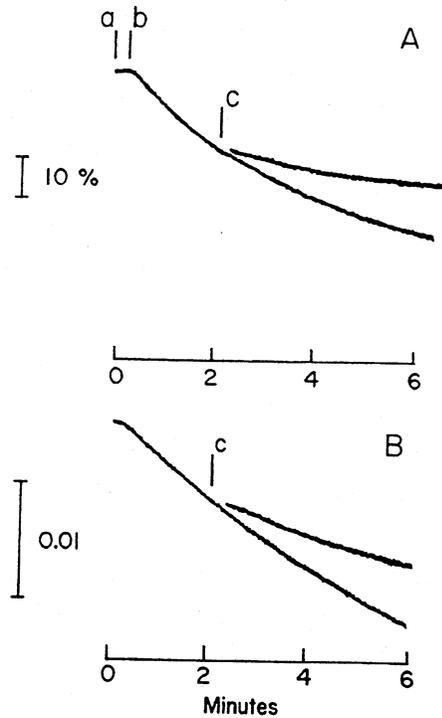
## RESULTS

The plasma membranes of corn root cells contained an electron transfer system which can use NADH to reduce either oxygen or ferricyanide (16, 17). Upon addition of NADH, RSO-plasma membranes catalyzed the reduction of both oxygen and ferricyanide as followed by changes in oxygen tension and absorbance at 420 nm, respectively (Fig. 1). The electron transfer rates to ferricyanide were much higher than those obtained for oxygen, 48 and 10.8  $\mu$ equivalent e-/mg protein/h, respectively. The addition of Al slowed the reduction of both oxygen and ferricyanide with little or no lag.

To gain further insight on the nature of the inhibition, RSO plasma membrane vesicles were treated with a range of Al concentrations (Fig. 2). Oxygen consumption rate decreased as the concentration of Al was increased. A similar decrease in the reduction of ferricyanide was observed.

Under the experimental conditions, the presence of Al had no detectable effects on either the absorption or fluorescence properties of NADH (data not shown). Thus, the possibility that Al affects the redox property of NADH is unlikely. Therefore, inhibition results from some interaction with the membrane *per se*.

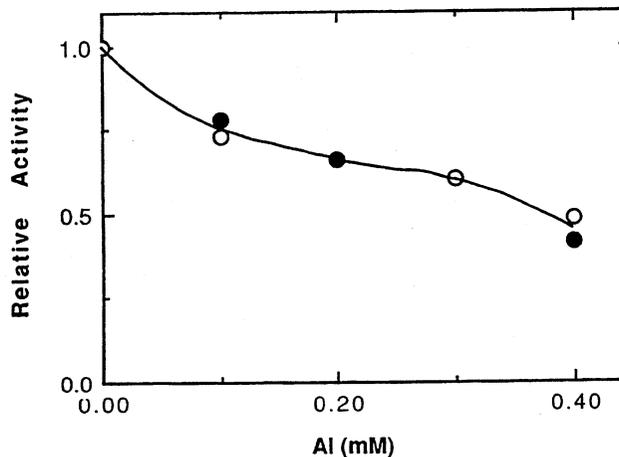
The next step in this investigation was to determine if inhibition was associated with a change in the structure of the lipid domain of the membrane bilayer. RSO plasma membrane vesicles were allowed to incorporate either DPH or DPH-PC. The degree of polarization of incorporated probes was evaluated at varying Al concentrations. Between 0 and 0.4 mM Al, little if any change in the polarization of either DPH or DPH-PC was observed. Within this range of Al concentration, polarization of DPH and DPH-PC averaged  $0.32 \pm 0.2$  and  $0.29 \pm 0.2$  (data not shown). When the Al concentration was increased above 0.4 mM, the degree of polarization increased markedly. This increase in polarization was associated with aggregation of the vesicles and their eventual precipitation.



**Figure 1.** Time Course of Oxygen (panel A) and Ferricyanide (panel B) Reduction by Right-side-out Plasma Membranes. Oxygen consumption was monitored after addition of 0.2 mg of membrane protein at point a followed by the addition of NADH at point b. Ferricyanide reduction catalyzed by 0.02 mg of membrane protein was monitored at 420 nm after the addition of NADH at time zero. The assay media was adjusted to a final concentration of 40  $\mu$ M Al at point c. The bar in panel a and b represents 10 % change in oxygen tension and 0.01 absorbance, respectively. The specific activity were 10.8 and 48  $\mu$ equivalent/mg protein/h for oxygen and ferricyanide reduction, respectively.

## DISCUSSION

The reduction of both ferricyanide and oxygen catalyzed by RSO plasma membranes from corn roots was inhibited by Al in a concentration dependent fashion (Figs. 1 and 2). This is the first report to our knowledge that Al can interfere with the reduction processes of the plasma membrane. It is likely that



**Figure 2.** Inhibition of NADH-linked Electron Transfer Catalyzed by Right-side-out Plasma Membranes. Vesicles were incubated with the indicated Al in assay media for 2 minutes before the addition of NADH. The specific activity in the absence of Al was 8.4 and 52  $\mu$ equivalent/mg protein/h for oxygen consumption and ferricyanide reduction, respectively. The standard errors of the mean did not exceed the size of the data symbols.

there is a common electron transfer step between the oxidation of NADH and the reduction of ferricyanide and oxygen, since both electron transfer activities were affected similarly by Al. However, the question whether the reduction site of oxygen is parallel to, in series of, or sharing an identical site with ferricyanide reduction remains to be determined.

The nature of inhibition of these reduction processes is yet to be determined. However, the mechanism does not seem to involve a direct interaction between Al and NADH since the intrinsic absorption and fluorescence properties of NADH were unaffected by Al. In addition, inhibition was not associated with a change in membrane structure as assayed by changes in polarization of fluorescence of either DPH or DPH-PC. Therefore, it would appear that Al inhibition of these redox reactions involves a direct interaction between Al and some component of the electron transport system. Alternatively, Al could be affecting the structure within distinct domains of the membrane that contain the electron transport system.

Changes in membrane structure within domains that comprise only a small portion of the membrane would not be detected by probes like DPH that measure the bulk membrane phase. Further research is needed to determine the mechanism by which Al interferes with electron transfer by plasma membranes.

**ACKNOWLEDGEMENT:** Mentioning of a brand or specific product does not constitute endorsement by U.S. Department of Agriculture.

**REFERENCES:**

1. Foy, C.D., Chaney, R.L. and White, M.C. 1978. The physiology of metal toxicity in plants. *Ann. Rev. Plant Physiol.* 29:511-566.
2. Godbold, D.L., Fritz, E. and Huttermann, A. 1988. Aluminum toxicity and forest decline. *Proceedings National Academy Science* 85:3888-3892.
3. Siegel, N. and Huang, A. 1983. Aluminum interaction with calmodulin. Evidence for altered structure and function from optical and enzymatic studies. *Biochimica Biophysica Acta* 744:36-45.
4. Wallace, S.U. and Anderson, I.C. 1984. Aluminum toxicity and DNA synthesis in wheat roots. *Agron. J.* 76:5-8.
5. Cho, S-W. and Joshi, J.G. 1989. Inactivation of Baker's yeast glucose-6-phosphate dehydrogenase by aluminum. *Biochemistry* 28:3613-3618.
6. Vierstra, R. and Huang, A. 1978. The effect of Al<sup>3+</sup> on the physical properties of membrane lipids in *Thermoplasma acidophilum*. *Biochemical Biophysical Research Communications* 84:138-143.
7. Tu, S-I. and Brouillette, J.N. 1987. Metal ion inhibition of corn root plasma membrane ATPase. *Phytochemistry* 26:65-69.
8. Nagahashi, G. and Baker, A.F. 1984. B-glucosidase activity in corn roots homogenates: problems in subcellular fractionation. *Plant Physiol.* 76:861-864.
9. Widell, S. and Larssen, C. 1981. Separation of presumptive plasma membrane from mitochondria by partition in an aqueous polymer two-phase system. *Physiologia Planterum* 51:368-374.

10. Clement J.D., Blein, J.P., Rigaud, J. and Scalla, R. 1986. Characterization of ATPase from maize shoot plasma membrane prepared by partition in an aqueous polymer two phase system. *Physiologie Vegetale* 24: 25-35.
11. Quail, P.H. 1979. Plant cell fractionation. *Ann. Rev. Plant Physiol.* 30: 425-488.
12. Buckout, T.J., Bell, P.F., Luster, D.G. and Chaney, R.L. 1989. Iron-stress induced redox activity in tomato (*Lycopersicum esculentum* Mill.) is localized on the plasma membrane. *Plant Physiol.* 90:151-156.
13. Brauer, D., Tu, S-I, Hsu, A-F., and Thomas, C.E. 1989. Kinetic analysis of proton transport by the vanadate-sensitive ATPase from maize root microsomes. *Plant Physiol.* 89:464-471.
14. Shinitzky, M. and Inbar, M. 1976. Microviscosity parameters and protein mobility in biological membranes. *Biochimia Biophysics Acta* 433:133-149.
15. Brauer, D., Schubert, C., Conner, D. and Tu, S-I. 1991. Calcium activation of maize root phospholipase. *J. Plant Nutri.* 14:729-740.
16. Lin, W. 1982. Responses of corn root protoplasts to exogenous reduced nicotinamide adenine dinucleotide: oxygen consumption, ion uptake, and membrane potential. *Proceedings National Academy Science* 79:3773-3776.
17. Blein, J. P., Canivenc, M. C. , DeCherade, X., Bergon, M., Calmon, J.P. and Scalla, R. 1986. Transplasma-membrane ferricyanide reduction in sycamore cells. Characterization of the system and inhibition by some phenyl biscarbamates. *Plant Science* 46:77-85