This is to certify that the

dissertation entitled

Aluminum Toxicity in Plants: A Potential Role for Organic Acids in the Prevention of Aluminum Induced Cellular Damage

presented by

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has been accepted towards fulfillment of the requirements for

Ph.D. degree in Botany & Plant Path.

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Date 5/16/86

ALUMINUM TOXICITY IN PLANTS: A POTENTIAL ROLE FOR ORGANIC ACIDS IN THE PREVENTION OF ALUMINUM-INDUCED CELLULAR DAMAGE

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1986

ABSTRACT

ALUMINUM TOXICITY IN PLANTS:
A POTENTIAL ROLE FOR ORGANIC ACIDS IN THE PREVENTION
OF ALUMINUM-INDUCED CELLULAR DAMAGE

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Aluminum-induced changes in the conformation, structure and function of calmodulin (CaM) and the corn root plasma membrane as well as the protective effects of aluminum (Al) ligands, especially organic acids against these changes were investigated by biochemical, enzymatic and spectroscopic techniques. The titration of CaM with Al ions results in a large increase in hydrophobic surface exposure and a substantial loss of alpha helix content of the protein at an [Al] to [CaM] molar ratio of 4:1. Ligands that were effective in protecting CaM from these Al-induced conformational changes were: fluoride ions and organic acids, especially citrate. When present in excess and prior to titration with Al ions citrate prevents Al-induced conformational changes in CaM. However, when Al was bound to CaM prior to citrate addition the hydrophobic domains but not the alpha helix content are restored to their original levels. Citrate chelation of Al from CaM is accompanied by a partial restoration of regulatory activity in CaM (80% of original activity) as judged by the phosphodiesterase enzyme assay. Citrate added in excess to CaM does not modify calcium-induced changes in both the hydrophobic surface domain and alpha helix content of CaM.

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Application of 10-50 μM concentrations of Al ions to a plasma membrane-enriched microsome fraction, isolated from roots of maize (Zea mays L.), resulted in decreased Mg^{2+}-ATPase activity, probably as a result of Al-induced changes in membrane structure, detected by the use of spin probes. Both enzymatic activity and membrane structure could in part be shielded from Al injury when organic acids, endogenous to maize root tissue, were administered prior to the metal. When stressed by application of Al ions, the Al-tolerant maize hybrid, W64, maintained higher concentrations of organic acids, especially malic and trans-aconitic, than the Al-sensitive maize hybrid, A632. The hypothesis that citric and malic acid, because of their high stability constants with Al and/or the acid's concentration reduce Al toxicity in maize root tissue, especially in the Al-tolerant line is presented.

Dedicated to Martha and Denes Suhayda.

ACKNOWLEDGEMENTS

I would like to thank the following members of my dissertation committee for their advice, help and guidance; Dr. Robert Bandurski, Dr. Alfred Haug, Dr. Estelle McGroarty, Dr. Derek Lamport, and Dr. Gerald Babcock. I owe special thanks to Dr. Robert Bandurski for serving as co-chairman of my committee and academic advisor and to Dr. Alfred Haug also serving as co-chairman who provided the advice, funding and research facilities necessary to complete this project.

I also wish to acknowledge Dr. Alan Saaf, Chairman of the Science Department at Lansing Community College, who provided me with the opportunity to serve as an instructor and experience the rewards of teaching.

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CHAPTER I

Literature Review

Introduction

Aluminum is one of the most abundant metals in the earth's crust, and is generally recognized as one of the most toxic upon its entry into the biosphere (1,2,3). The presence of aluminum in soil and subsoil coupled with the enhanced mobility of this metal under acidic conditions presents aluminum toxicity as a formidable barrier to successful food and biomass production in a substantial portion of the world's arable soils (4). Unfavorable physicochemical environmental conditions including water availability, acidity, alkalinity, mineral toxicity, and salinity have been estimated to depress crop yields by 71 percent (5). Aluminum contributes directly to this reduction in crop productivity by its phytotoxic effects on plants and indirectly by limiting root development and penetration into subsoils thus enhancing the drought susceptibility of crops and limiting their access to mineral nutrients (2). In addition to the naturally occurring problem of soil acidity the atmospheric deposition of mineral acids (acid rain) onto the lakes and soils of the northeast U.S. and Canada has led to the transport of labile monomeric aluminum ions in the soil and enhanced the concentration of this metal ion in lakes and surface waters (6,7). A recent hypothesis formulated to explain the apparent acid rain related dieback of European forests suggests that mobile Al ions exert their toxic effects on the fine roots of afflicted trees resulting in significant losses in root structure and the eventual dieback of the aerial portions of these trees (8). This model for the action of acid rain-induced Al toxicity was developed for mineral soils whereas

available data from a typic Haplorthod soil containing moderate amounts of organic matter show very little free aqueous Al being present with most Al complexed to organic matter (7). A recent study of plants exposed to aluminum ions and simulated acid precipitation attributed the cessation of plant growth to calcium deficiency resulting from aluminum inhibition of calcium uptake and translocation (9).

Since aluminum toxicity is a soilborne problem the development of aluminum tolerant plants requires the identification of the primary target(s) of aluminum ions in root cells and an understanding of the mechanisms by which this metal ion exerts its toxic effects on the physiological and biochemical processes of roots. The elucidation of these pathways and potential plant defense mechanisms coupled with sensitive screening assays (10) will aid in the selection of aluminum tolerant crop varieties. It is my aim in this dissertation to investigate the toxic effects of aluminum ions at the cellular and subcellular levels and to identify potential biochemical markers for aluminum tolerance mechanisms of cells.

The Physiology of Aluminum Toxicity in Plants.

The root is a specialized plant organ that supplies the above ground portions of the plant with nutrients and anchors it in the soil. Thus a plant root has three primary functions: to absorb water and nutrients from the soil, to provide the upward transport of water and nutrients to the above ground regions of the plant, and to transport the products of photosynthesis from the aerial portions of the plant to the roots (11). The root has the capacity to concentrate metals from the soil solution up to 10,000-fold in excess of that in the surrounding

environment by both active and passive transport processes (11). The concentration of Al in soil solution is pH dependent and usually does not exceed 150 μM (4 ppm) (1). Simulated acid rain leaching of three Delaware soil types has shown a pH dependence of Al release that ranged from 0.74 to 25 μM at pH 2.5 and 5.6, respectively (12). It is generally accepted that in plants cultured in nutrient solution the concentrations of Al must be in excess of 10 μM in order for symptoms of Al-induced phytotoxicity to appear (13).

The primary target organ of Al toxicity is the root. In corn roots the primary site of Al uptake seems to involve cells on the periphery of the root cap (23). Al prevents proper root development and thus the penetration of roots into the subsoil which limits the availability of nutrients and water to the plant (14). Morphological changes are readily observable in Al-stressed roots. These manifest themselves in: the stunting of the main root, thickening and brownish discoloration of lateral roots and root hairs, and the lack of root branching (14). Interestingly enough, the symptoms of Al toxicity are those characteristic of the deficiency of several physiologically important ions including: calcium (2,14), phosphorus (2,14), and iron (16). Of these, Al-induced calcium deficiency symptoms are the most striking (2,9). The protective effects of calcium in the alleviation of Al toxicity have been documented with whole plant (17), solution culture (10,18), and cell suspension culture (19) studies. The maintainence of root function by calcium in the presence of environmental stress has been attributed to the complexing of this ion with oxygen containing ligands in the cell wall and plasma membrane thereby

displacing toxic cations from these sites, and thus maintaining cell wall integrity and selective membrane permeability (20). At the cellular level Al interferes with the uptake of essential elements such as Mg, Ca, P, Mn, Zn, and Fe (14,15,24). Aluminum crosslinks acidic components of the cell wall, disrupts the activity of wall localized metabolic enzymes (21,14) and may play an important role in the observed reduction in cell division (22).

Aluminum Tolerance Mechanisms in Plants.

When stressed with aluminum, Al-tolerant varieties of barley, soybean and wheat accumulated less of this metal in their roots than the respective Al-sensitive cultivars (5). Ryegrass, wheat, and barley that are Al-tolerant in some cases accumulate less Al in their tops than susceptible cultivars (25). The observed differential tolerance to Al by plant cultivars is most likely the consequence of the existence of an effective defense mechanism(s). Al-accumulating plants have the ability to concentrate high levels of Al (some in excess of 1000 ppm, 1 ppm = 37 μM) in their tissues without any apparent symptoms of Al toxicity. Some of these plants include tea (26) and mangrove (25) and certain dicots of tropical rainforest families (27). Two potential strategies for the detoxification of Al by plants appear to be especially feasible: first, the prevention or the reduction of Al entry into the root and second, the complexation or compartmentation of Al upon entry into the plant so as to limit Al-induced cellular damage. Consistent with the first strategy, certain Al-tolerant plant cultivars can increase the pH of the soil solution that is in intimate contact with the root (25,28,29) whereas Al-sensitive cultivars lower the pH of the root zone.

Although this mechanism may reduce the Al uptake into plant roots the fact that high concentrations of Al have been found associated with subcellular fractions of Al-tolerant wheat varieties implies that other resistance mechanisms must be operative (33). With respect to the latter mechanism it appears that Al-tolerant plants can complex this metal with various organic acids (30,31,32) and Al-citrate complexes have been identified in the heartwood of an Adinandra brassii tree growing on bauxite containing soils (34). Organic acid complexation of Al by Al-tolerant plants was first suggested as a mechanism to prevent Al-induced precipitation of phosphorus in the cell (35). Acid tolerant plants appear to possess an intracellular buffering system based on organic acids whereas alkaline tolerant plants possess a cytoplasmic buffering system based on phosphate (36). Conceivably the organic acid based cellular buffering system could render these plants more Al-tolerant. This is consistent with numerous experimental observations made on plants subjected to Al-stress. Corn plants cultured hydroponically on nutrient solution supplemented with Al salts developed symptoms characteristic of Al-toxicity that coincided with a redistribution of physiologically important ions throughout the various tissues (24). However, when Al was provided as either the citrate or EDTA chelate the plants did not manifest the changes typical of Al-toxicity at either the whole plant or tissue levels. Experiments with freshly germinated red clover seedlings also showed that the addition of 10 μM citrate to a solution culture containing 1 μM Al ions completely ameliorated the inhibitory effects of Al on root growth (10). These observations have also been confirmed with tissue cultured plant cells. A carrot cell line selected for Al-tolerance produced citrate in excess

and released it into the culture medium. Furthermore, it solubilized the Al which had originally precipitated in the gel as Al hydroxide and Al phosphate (32). Similarly, tobacco cells supplemented with 800 μM ionic Al in the culture medium showed a 70% reduction in cell fresh weight after 10 days whereas cells supplemented with the same level of Al complexed with citrate had fresh weights comparable to control cells (19). These observations suggest a protective role for citrate as well as other naturally occurring organic acids against the toxic consequences of Al ions. The observed protective effects of citrate against Al toxicity can probably be attributed to the strong, stable complex that it forms over the physiological pH range of 5-8 as judged by ^{27}Al NMR (37) and a stability constant of $5.0 \cdot 10^{8}$ M^{-1} (38). Malate, with a stability constant of $7.2 \cdot 10^{5}$ M^{-1} for Al (38) may also be pivotal in providing protection against Al toxicity.

Organic acid-Al complexes may provide a mechanism for the distribution of Al ions in the plant. The role of citrate in iron transport and metabolism by plants is better defined and by analogy may provide some insight into the transport of Al-citrate complexes in the plant. The Fe-citrate chelate has been identified in the xylem exudate of sunflower, soybean, cucumber and tomato (39,40,41). Soybean plants provided with Fe-EDDHA, a synthetic Fe chelate, in solution culture were found to possess only the Fe-citrate complex in their xylem exudate. This metal is presumably transported and translocated throughout the plant as a chelate of citrate. Variations in the citrate/Fe ratio in xylem exudate were found to be sensitive to the external Fe concentration of the culture media. Chlorotic sunflower plants cultured on a low Fe media absorbed large quantities of Fe from the

external medium and translocated it in xylem exudates at concentrations well above the external medium (actual citrate and Fe concentrations in the exudate were 890 μM and 310 μM, respectively). Control sunflower plants grown with adequate Fe in the media showed a controlled uptake of iron and, in fact, excluded most of the iron supplied to the roots (actual citrate and Fe concentrations were 30 μM and 2 μM, respectively in the exudate). Analysis of xylem exudates revealed that the chlorotic plants had a citrate/Fe ratio of 3 in contrast to control plants with citrate/Fe ratio of 15 (40). It was suggested that the large molar excess of citrate may be involved with the solubilization and transport of other metals. These findings raise the possibility that Al may be solubilized and transported in the plant by a similar mechanism and may represent a way for Al ions to interfere with or disrupt essential metal metabolism in plants.

The subcellular compartmentation of Al into the vacuole may be a viable means for limiting the cellular toxic effects of this metal. ^{31}P NMR measurements performed on corn root tissue exposed to 5 mM Al ions in the presence of 50 mM glucose provided indirect evidence via the loss of the inorganic phosphorus signal that Al ions had been shuttled into the vacuole (42). In this same study the transport and compartmentation of excess manganese from the cytoplasm into the vacuole under aerobic conditions was also demonstrated.

Finally, there exists some evidence for the induction of an Al binding protein(s) when plant roots are preincubated with sublethal concentrations of Al ions in the culture medium (43). In this study wheat seedlings preincubated for 48 h with 0.1-0.5 μg/ml Al solutions

and subsequently challenged with 2-12 µg/ml Al solutions had consistantly better root growth than the non-pretreated controls. The addition of cycloheximide to the Al pretreatment solutions completely negated the growth differential between Al pretreated and non-pretreated wheat seedlings. These Al pretreatment periods were found to overlap with significant increases in the incorporation of ^{14}C-valine and ^{3}H-thymidine into trichloroacetic acid precipitates of wheat root homogenates suggesting protein synthesis in response to Al stress.

Aluminum-Membrane Interactions

Aluminum-membrane interactions may be one of the primary factors responsible for Al toxicity in cells. Ultrastructural studies of the primary root of Zea mays L. have shown that upon Al treatment (840 µM) the initial point of entry of Al ions into root tissue occurs at the peripheral cells of the root cap and the mucilage surrounding the root (23). While Al ions rapidly translocated through cells of the root cap they were not detected in primary root meristem during the initial 20 h of Al treatment. The initial subcellular changes associated with Al uptake by root cap cells were disruption of the migration of secretory vesicles from dictyosomes to the plasma membrane, disruption and alteration of the dictyosome membrane and alterations in the endoplasmic recticulum membrane (44). The accumulation of lipid droplets in plastids was postulated to be an indication of Al interference with membrane assembly (45). In a comparable ultrastructural study with Zea mays L. cultured in 100 µM lanthanum, a trivalent cation, this ion was accumulated in the first 0.5 cm of root tissue from the apex where it

was associated with vesicles in the cytoplasm that were in close proximity of vascuoles while additional deposits of lanthanum were observed in the endoplasmic recticulum (46). These observations in conjunction with ^{31}P NMR measurements that provide indirect evidence for Al accumulation in the cytoplasm and vacuole of corn root tissue (42) provide a strong indication for active Al uptake by cells with a close interaction between Al ions and cellular membranes.

Al-induced membrane damage may be the consequence of Al ion perturbation of lipid bilayer physical properties. Al interactions with liposomes containing fluorescent labelled phospholipid analogs have shown that upon titration with 30-100 μM Al ions the phospholipids undergo phase separation which was coincident with liposome aggregation as well as increased liposome permeability (47). These effects were attributed to the strong interaction between Al ions and phosphatidylserine (48), estimated to have a dissociation constant of 10^4-10^5 M^{-1} (49). This was reinforced by the observation that increasing the relative molar concentrations of phosphatidylcholine or phosphatidylethanolamine to that of phosphatidylserine attenuated all of the above Al-induced effects, and Al-addition to neutral membranes containing phosphatidylethanolamine did not result in lipid phase separation (49). Al ions were able to displace ^{45}Ca ions from the liposome surface and the preaddition of millimolar amounts of Ca ions afforded some protection against titration with Al ions. Al apparently forms stable complexes with phosphatidylserine since the addition of excess citrate to Al-treated liposomes could not completely reverse Al-induced phase separation (47,49). The Al perturbation of membrane fluidity in

Thermoplasma acidophilum has been measured by electron spin resonance spectroscopy (50). This study revealed that Al concentrations as low as 10 μM produced pronounced phase changes in the plasma membrane of this organism. In addition to the direct effect of Al on membrane lipid physical properties the ion has been shown to aid in the peroxidation of lipids by iron salts (51). Lipid peroxidation by Cu^{2+} ions in the presence of H_2O_2 has also been demonstrated; it should be noted that the addition of EDTA or citrate inhibited this metal catalyzed lipid peroxidation (52).

Aluminum Interactions with Membrane-Bound ATPase and Calmodulin.

Plant membrane-bound ATPases via the pumping of protons are responsible for the generation of membrane potential and pH gradient differences across cell membranes (53). The movement of solutes across the cell membrane occurs down energy gradients created by these proton fluxes. Al inhibition of ATPase activity could therefore disrupt proton transport and in turn affect the transport of other solutes. Recent evidence indicates that the complexation of Al by ATP when preincubated with the nucleotide at Al concentrations above 100 μM can inhibit basal Mg^{2+}-dependent ATPase activity from barley roots by up to 50% (54). It is possible that at lower Al concentrations when Al is incubated directly with plasma membrane preparations the ion may inhibit the ATPase by acting upon enzyme associated membrane lipids, by changing the conformation of the enzymatic protein upon binding as demonstrated for heavy metals (55), or by interacting with proteins that regulate ATPase activity (56). The calmodulin-dependent formation of the transmembrane potential in plasma membrane vesicles isolated from

barley roots was diminished by the stoichiometric addition of Al ions to the enzyme system containing added calmodulin (56). The addition of equivalent amounts of Al ions to membrane vesicles in the absence of calmodulin failed to affect the transmembrane potential suggesting the direct effect of Al ions on calmodulin activity. Electrophysiological measurements employing root tissue have also shown that the addition of micromolar quantities of Al ions to root cells lowered the transmembrane potential (57), however since these measurements were performed on whole cells the mechanism of Al action could not be determined.

It is evident from the available literature regarding Al toxicity in cells that both the plasma membrane and the calcium binding regulatory protein, calmodulin represent key targets for Al ions in the cell. This dissertation will be focused on further characterizing Al-induced structural and functional changes in the root cell plasma membrane and calmodulin. The effects of strong Al ligands on Al interactions with these cellular components will be examined in order to identify possible Al tolerance mechanisms in cells. These studies will be performed using biochemical, enzymatic and spectroscopic techniques.

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CHAPTER II

Metal-Induced Conformational Changes In Calmodulin

Introduction

A molecular basis for metal-induced alterations in cellular metabolism leading to the expression of metal toxicity symptoms and potential cell death has not been elucidated. An attractive hypothesis relating to the possible mode of action of toxic metals would be that their primary target within the cell is a key regulatory protein. The potential consequences of the activation or deactivation of a regulatory protein by metals could be a cascading effect with numerous biochemical processes being affected and in turn profoundly altering cellular metabolism. One potential target for toxic metals found in all eukaryotic cells is the metal binding protein, calmodulin (CaM), an acidic 17,000 mw protein possessing four calcium (Ca^{2+}) binding domains. This protein has a multitude of regulatory functions in the cell including the regulation of: cyclic nucleotide metabolism, the Ca^{2+} transport ATPase of the red blood cell, cell motility including the depolymerization of microtubles, protein phosphorylation, etc. (1). The regulatory activity of CaM is governed by the levels of free intracellular Ca^{2+} which are estimated to range from 10^{-7} M for various types of unstimulated cells to 10^{-5} M for stimulated cells (2). Regulation of enzyme activity by CaM is the end product of a reversible two step process (3). The first step involves the binding of Ca^{2+} ions to CaM thereby inducing conformational changes in the protein that are characterized by increases in the surface hydrophobicity and alpha helix content, and resulting in the formation of an activated CaM molecule. This is followed by the binding of the activated Ca^{2+}-CaM complex to a specific site on the target enzyme with an ensuing CaM-

induced conformational change in the enzyme reflected by an alteration in enzymatic activity.

The aim of this study is to investigate the potential for metals other than Ca^{2+} to activate CaM by inducing the required conformational changes in the protein. An attempt is made to establish a relationship between metal ion-induced conformational changes in CaM and chemical and physical properties of the metal ions such as valency and ionic radius. The surface hydrophobicity and alpha helix parameters were measured since these conformational changes are involved in the formation of the activated CaM complex upon Ca^{2+} binding.

Materials and Methods

Fluorescence and circular dichroic measurements were recorded as previously described (4). The hydrophobic fluorescence surface probe 8-anilino-naphthalene sulfonic acid (ANS) was used as the sodium salt to monitor changes in the hydrophobic domains of CaM. ANS has been previously used to characterize Ca^{2+}-induced conformational changes in CaM (5). Fluorescence measurements were made in 10 mM Pipes buffer, pH 6.5 at ANS and CaM concentrations of 2 µM and 10 µM, respectively. Circular dichroic spectra were recorded using a CaM concentration of 10 µM in 10 mM Tris/HCl buffer at pH 6.5. All spectroscopic measurements were made at $22^{o}C$.

CaM was isolated by a combination of ion-exchange and affinity chromatography techniques as previously described (6). The CaM stimulated activity of the cyclic GMP-dependent phosphodiesterase was measured in accordance with previously published techniques (6).

Results and Discussion

The fluorescent dye ANS has been used to monitor changes in hydrophobic surface sites on CaM in response to metal titration. ANS binds to CaM in a calcium-dependent manner with a K_d of 1 mM for 2.8 ANS binding sites (5). In the presence of Ca^{2+} ions ANS prevents CaM binding to phosphodiesterase suggesting that this fluorescent ligand interacts with the hydrophobic domain on CaM which serves as it's interface with target enzymes (5). The data in Table 1 show the enhancement of ANS fluorescence of the dye-protein complex in response to metal ion titration. The alkali cation Li^+ with an ionic radius of 0.68 Å did not induce a substantial change in ANS fluorescence when titrated to a Li^+/ CaM molar ratio equal to 8. This suggests that Li^+ cannot induce the conformational changes required for development of the hydrophobic domain needed to form the activated metal-CaM complex. Since this monovalent ion failed to produce any changes in the fluorescence of the dye-CaM complex the effects of Li^+ on the alpha helical content of CaM was not examined. Titration of CaM with Cu^{2+} ions under identical conditions resulted in a slight suppression in the fluorescence intensity of the ANS-CaM complex (Table 1). Cu^{2+} ions by their interaction with or binding to CaM apparently reduce the hydrophobic surface domains on the protein perhaps indicating a decrease in the number of ANS molecules bound to CaM. A 1% reduction in the alpha helix content of CaM was observed when Cu^{2+} ions were added to the protein to a molar ratio of Cu^{2+} / CaM equal to 8. The seeming lack of interaction between Cu^{2+} ions and CaM may in part be explained by the physico-chemical properties of this ion. Cu^{2+} has a nonhydrated ionic radius of 0.72 Å providing the ion with a charge to

TABLE 1. Cation-induced changes in relative ANS fluorescence intensity and alpha-helix content of bovine brain calmodulin.

[Metal]/[CaM] (mol / mol)	Metal	Change in Relative ANS Fluorescence	% α-Helix
0	-	-	32.0
2	Li^{+}	+0.03	nd
4	Li^{+}	+0.06	nd
8	Li^{+}	+0.06	nd
2	Cu^{2+}	-0.03	32.6
4	Cu^{2+}	-0.25	32.6
8	Cu^{2+}	-0.72	31.4
2	Y^{3+}	+0.27	33.6
4	Y^{3+}	+1.73	34.4
8	Y^{3+}	+2.57	28.2
2	Zr^{4+}	+0.40	31.6
4	Zr^{4+}	+0.73	31.6
8	Zr^{4+}	+1.43	28.4

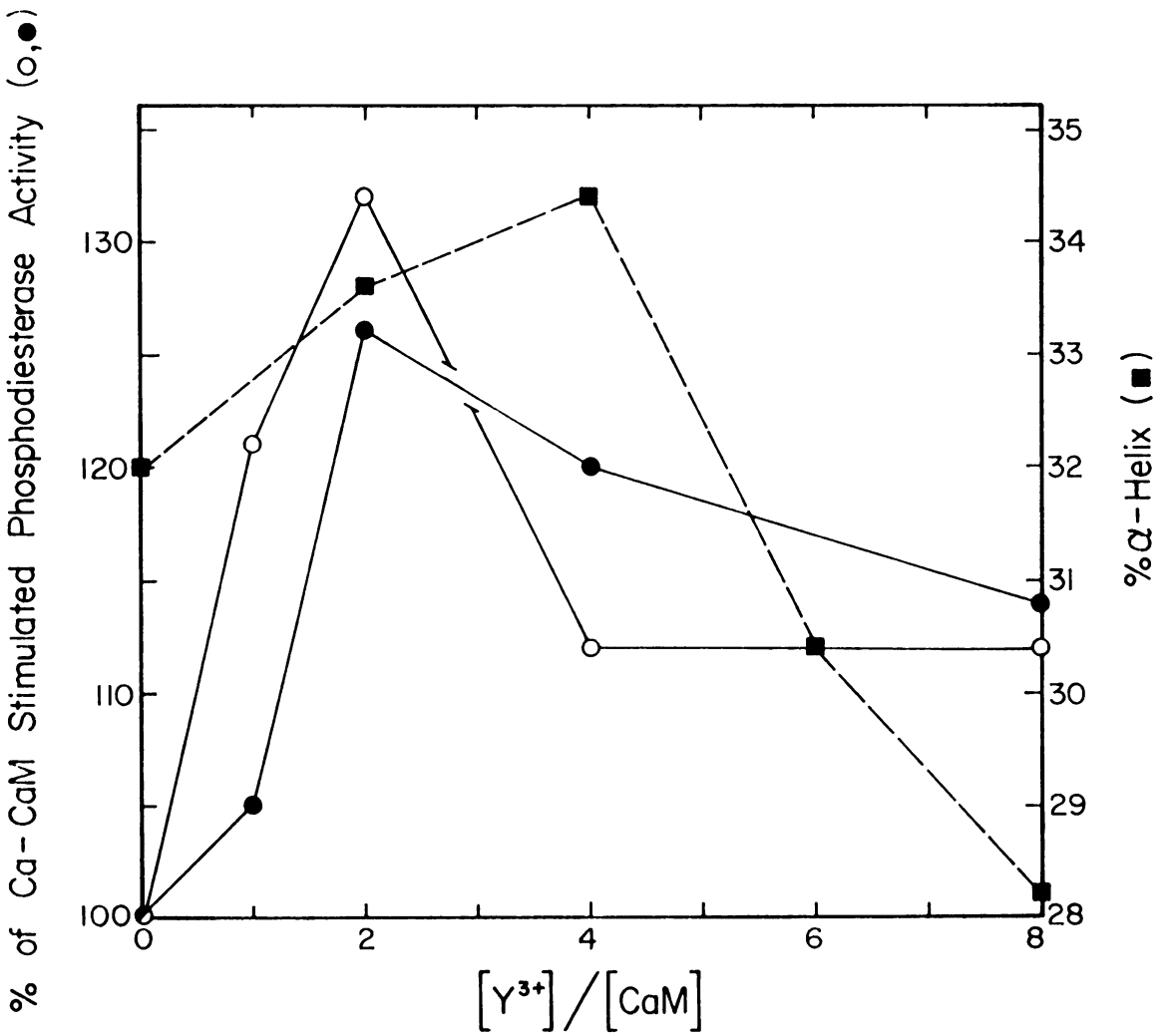
Note: For ANS fluorescence measurements bovine brain CaM and ANS dye were used at concentrations of 10 μM and 2 μM, respectively at pH 6.5 in 10 mM Pipes buffer. For the circular dichroic measurement of mean residue ellipticity from which alpha helix content was calculated the concentration of CaM was 10 μM in 10 mM Tris buffer, pH 6.5.

size ratio of 2.78. In comparison Ca^{2+} ions have a nonhydrated ionic radius of 0.99 Å and a corresponding charge to size ratio of 2.02. Potentially the smaller size of the Cu^{2+} ion in conjunction with its higher charge density as compared to Ca^{2+} ions make it unsuitable for coordination in the Ca^{2+}-binding domains of CaM.

Trivalent yttrium ions, Y^{3+} titrated onto CaM increased the relative ANS fluorescence of the dye-CaM complex only slightly at a molar ratio of Y^{3+} / CaM equal to 2 (Table 1). At this same ratio there was a 1.6% increase in the alpha helix content of the protein over that found for metal free CaM. At a metal to CaM molar ratio of 4 the increase in ANS fluorescence (+1.73) paralleled the 2.4% increase in the alpha helix content of the protein. However at the highest molar ratio of Y^{3+} / CaM used, 8, the increase in fluorescence intensity (+2.57) was accompanied by a sharp decline of 6.2% in the alpha helix content of CaM from that found at the Y^{3+} / CaM molar ratio equal to 4. Since the initial observed conformational changes in CaM upon Y^{3+} addition resembled those induced by Ca^{2+} ions (6) the Y^{3+}-CaM complex was assayed for regulatory activity using the CaM stimulated cGMP-dependent phosphodiesterase (PDE) assay (Figure 1). In this assay Y^{3+} was added in stoichiometric amounts to metal free CaM (concentration 0.2 μM) in the absence or presence of saturating levels of Ca^{2+} ions (concentration 50 μM) and the Y^{3+}-CaM stimulated phosphodiesterase activity was measured at pH 7.0. The basal PDE activity in the absence of Ca^{2+} ions and CaM was 1.4 nmol cGMP hydrolyzed $\cdot$ ml^{-1} $\cdot$ min^{-1} and the 100% activity corresponds to that measured in the presence of 0.2 μM CaM and 50 μM Ca^{2+} ions and having an activity of 1.8 nmol cGMP hydrolyzed $\cdot$ ml^{-1} $\cdot$ min^{-1}. The data show that the addition of 1 Y^{3+}

Figure 1. Stimulation of cGMP-dependent phosphodiesterase by the Y^{3+}-CaM complex and its relationship to the Y^{3+}-induced change in alpha helix in CaM.

The Y^{3+}-CaM stimulation of the enzyme was measured at pH 7.0 in the absence (○) and presence (●) of 50 μM Ca^{2+} ions. The concentration of bovine brain CaM was 0.2 μM and Y^{3+} was added at the respective molar ratios indicated on the abscissa. The basal activity of the enzyme measured in the absence of Ca^{2+} ions and CaM was 0.14 nmol cGMP hydrolyzed · ml^{-1} · min^{-1}. The 100% value corresponds to the maximum Ca^{2+}-CaM stimulated enzyme activity of 0.18 nmol cGMP hydrolyzed · ml^{-1} · min^{-1} when the respective Ca^{2+} and CaM concentrations used were 50 μM and 0.2 μM. The Y^{3+}-induced change in the alpha helix content of CaM (■) was measured at pH 6.5 in Tris buffer using CaM at a concentration of 10 μM.


% of Ca-CaM Stimulated Phosphodiesterase Activity (o,●)
%α-Helix (■)
[Y3+]/[CaM]
100
110
120
130
28
29
30
31
32
33
34
35
0
2
4
6
8

ion per CaM molecule results in a stimulation of PDE activity that is 20% greater than that observed with Ca^{2+}-CaM. Increasing the metal content of the protein to 2 Y^{3+} ions per CaM molecule stimulated PDE activity 32% above the Ca^{2+}-CaM activity. However the further addition of Y^{3+} ions to CaM up to a molar ratio of 8 resulted in a reduction in Y^{3+}-CaM stimulation of PDE to a level approximately 12% above the observed Ca^{2+}-CaM stimulation of the enzyme. Similar effects, although attenuated, were observed when Y^{3+} ions were added to the Ca^{2+}-CaM complex (Figure 1). Although the increase in alpha helix content of CaM upon Y^{3+} titration parallels the initial observed increase in regulatory activity of the Y^{3+}-CaM complex the helix content of the protein continues to increase up to an Y^{3+} / CaM molar ratio of 4 whereas at this metal concentration there is a sharp decline in Y^{3+}-CaM stimulated PDE activity. It is apparent that neither the ANS fluorescence intensity increase nor the alpha helix content of CaM, the two parameters monitored to detect conformational changes in the protein, directly coincide with the observed changes in Y^{3+}-CaM stimulated PDE activity. The data on Y^{3+}-CaM interactions shows that the binding of 1 Y^{3+} ion to CaM will induce the required conformational changes in the protein to form an activated Y^{3+}-CaM complex capable of interacting with target enzymes. It appears that Y^{3+} ions bind to CaM with a higher affinity than Ca^{2+} ions. This is exemplified by stimulation of PDE activity above the level found for Ca^{2+}-CaM stimulation of PDE when Y^{3+} was added to Ca^{2+} saturated CaM (Figure 1). Similar effects for Tb^{3+} activation of CaM in the absence of Ca^{2+} when added up to a Tb^{3+} / CaM molar ratio equal to 3 were observed with respect to PDE stimulating activity (7). However in this report stimulation of PDE by the

Tb^{3+}-CaM complex did not exceed the maximal stimulation observed with Ca^{2+}-CaM. Studies on the Ca^{2+}-induced conformational changes in CaM have shown that upon the binding of the first two Ca^{2+} ions a hydrophobic plate is exposed on the protein's surface that allows interaction with the hydrophobic dye, 2-p-toluidinylnaphthalene-6-sulfonate (TNS), but does not convert CaM to an active form capable of enzyme regulation (8). Activation of CaM is achieved by the binding of a third Ca^{2+} ion to CaM (8). Available data indicate that the third Ca^{2+} ion binds to a region of CaM corresponding to amino acid resides 80-113. Upon ion binding this region increases in alpha helix content which is thought to expose and stabilize the hydrophobic domain formed upon the binding of the first two Ca^{2+} ions (9). A possible explanation for the observed Y^{3+} activation of CaM could be that the first one or two Y^{3+} ions binding to CaM interact at the region corresponding to amino acid residues 80-113 resulting in the simultaneous formation and stabilization of the hydrophobic plate required for CaM interaction with target enzymes. It should be noted that the Y^{3+} nonhydrated ionic radius is 0.92 Å, approximately that of Ca (0.99 Å). Perhaps this similarity in size allows for Y^{3+} ions to interact with the metal binding domains of CaM which have evolved to accommodate the Ca^{2+} ion. In solution only the trivalent state of yttrium is stable and the ion coordinates 9 water molecules in its primary hydration shell (10). The solution chemistry of Y^{3+} is complex with the commonly occurring cationic hydrolysis products being YOH^{2+}, $Y_2(OH)_2^{4+}$, and $Y_3(OH)_5^{4+}$ (10). It is not known whether one or more of these species interacts with CaM in solution.

Interactions of Zr^{4+} ions with CaM when added to a Zr^{4+} / CaM molar ratio of 8 resulted in increased fluorescence of the dye-CaM complex and a decrease in the alpha helix content of the protein (Table 1). This resembles the aluminum effect on CaM although lower aluminum concentrations can be used to obtain changes of the magnitude observed with Zr^{4+} (6). The disruption of helix structure in the protein apparently results in the appearance of hydrophobic domains on CaM. The observed Zr^{4+} effects may be related to the solution chemistry of the tetravalent ion. In dilute aqueous solution the ion exists as the following hydroxide complexes: $Zr(OH)_5^-$, $Zr(OH)_4$, and $Zr(OH)_3^+$ occurring at 55%, 35%, and 5%, respectively of the total ion species in solution at pH 6.5 (10). The charge density of Zr^{4+} is 5.06 in comparison to Al^{3+} which has a value of 5.88. Possibly Zr^{4+} and Al^{3+} ions act via a similar mechanism in the disruption of CaM conformation.

In summary there appears to be a correlation between the ionic radius of a metal (as in the case of Y^{3+}) and its ability to induce conformational changes in CaM that result in activation of the protein. Apparently the Ca^{2+} binding domains of CaM are tailored to accommodate ions having a radius similar to Ca^{2+}. The Zr^{4+} ion has been identified as a metal that can disrupt CaM conformation similar to Al^{3+} ions. The Zr^{4+}-induced conformational change may interfere with the regulatory activity of CaM. There appears to be a correlation between high charge density for a metal ion and its ability to disrupt the alpha helix content of CaM. Thus the possibility exists that under the appropriate conditions metal ions other than Ca^{2+} may activate or deactivate CaM and in turn profoundly affect cell metabolism.

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CHAPTER III

Aluminum Changes The Conformation Of Calmodulin

Physiol. Chem. & Physics 14 *(1982)*

ALUMINUM CHANGES THE CONFORMATION OF CALMODULIN

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• *Fluorescence titration experiments indicate that Al^{3+} binds stoichiometrically to electrodialyzed bovine brain calmodulin. There are three binding sites for aluminum on the protein. Application of Al^{3+} to calmodulin appears to expose larger hydrophobic surface domains as compared with those found for calmodulin in the presence of calcium or gallium.*

Calmodulin is a ubiquitous and multifunctional calcium-regulating protein which has been shown to participate in a variety of calcium-dependent processes including stimulation of ATPases and phosophodiesterases[1]. Calmodulin has a molecular weight of about 17,000 and has been conserved during evolution[2]. Dependent upon the ionic strength, the dissociation constants of the four calcium binding sites lie in the micromolar range[3]. In the case of bovine brain calmodulin, the high affinity sites I and II are devoid of tyrosyl residues, while the low affinity sites III and IV are associated with one tyrosine each, *viz.*, tyr 99 at site III, and tyr 138 at site IV[1,4]. These are the sole tyrosine residues in the entire calmodulin molecule, tryptophan residues are lacking. Therefore measurements of tyrosine fluorescence, or of energy transfer from tyrosine to luminescent lanthanides, can be performed to investigate metal-induced conformational changes of calmodulin[4,5]. These changes generate domains with considerable hydrophobicity as evidenced by experiments employing hydrophobic fluorescence probes like 8-anilino-1-naphthalene sulphonate, ANS[6].

Aluminum accumulation in animals and man has been implicated in diseases like Alzheimer's disease and dialysis dementia[7]. In plants, micromolar concentrations of aluminum in the soil decreased the rate of root elongation and induced symptoms typical of calcium deficiencies[8]. Considering the importance of calmodulin in calcium regulation, the potent interaction of aluminum with calmodulin shown to occur in this study may represent a crucial biochemical lesion of aluminum toxicity.

Calmodulin was prepared from bovine brain acetone powder and purified by phenothiazine affinity chromatography[9]. The eluted calmodulin was dialyzed against distilled water, electrodialyzed and then lyophilized. The protein activated 3':5'-cyclic nucleotide phosphodiesterase and migrated as a single band during sodium dodecyl sulphate gel electrophoresis. Fluorescence intensity measurements were carried out on a Perkin-Elmer spectrofluorimeter, model MPF-44A, equipped with a differential corrected spectra unit. The ANS fluorescence intensity of calmodulin, in the absence of metal, was considered as the initial fluorescence intensity value. Data for ANS fluorescence, in the presence of the metal, are expressed as relative increase in the initial fluorescence intensity value. Each value represents the mean of at least three separate calmodulin preparations within 5% standard error.

Application of aluminum to calmodulin seems to expose larger hydrophobic surface domains as compared with those found in the presence of calcium or gallium. At a metal concentration of about 25 μM, calcium enhanced the ANS fluorescence intensity by about 2%, whereas gallium and aluminum increased the intensity by about

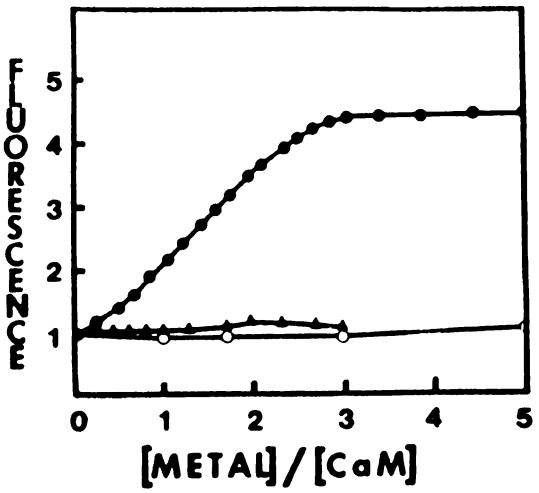

FIGURE 1. ANS fluorescence of bovine brain calmodulin as a function of [metal]/[protein] (mol:mol) ratio. A 7 μM concentration of calmodulin was prepared in 10 mM morpholino propane sulphonic acid (MOPS) buffer, pH 6.5. The concentration of 8-anilino-1-naphthalene sulphonate, ANS, was 2 μM. Excitation wavelength was 360 nm, ANS fluorescence intensity was recorded at 490 nm. The ordinate lists the metal-induced relative fluorescence intensity. The protein was titrated with the metal ions, *viz.* Al^{3+} (●), Ga^{3+} (▲), and Ca^{2+} (○).

20 and 400%, respectively (Fig. 1). The ANS fluorescence titration curve reached a maximum at a ratio of 3 mol of Al^{3+} per mole of calmodulin. Our findings therefore indicate that aluminum binds to calmodulin in a stoichiometric manner. Hill plots suggest that the affinity of aluminum to calmodulin is at least one order of magnitude larger than that of calcium to calmodulin. It seems worth noting that aluminum and the closely related gallium have similar binding constants. As calculated by the procedure of Chen et al.[10], results from our circular dichroism studies indicate that Al^{3+} application to calmodulin decreases the α-helical content of the protein in contrast to the observed increase of α-helix content upon binding of calcium in the protein[1](Table I). Upon binding calcium, specific changes in the internal protein structure allow calmodulin to participate in the second messenger system[3]. Loss of this structure occurs in the presence of aluminum as shown by our studies. We propose that the loss of this structure probably impairs the functioning of calmodulin as a calcium regulator.

TABLE I. α-Helical Content of Bovine Brain Calmodulin in the Presence of Al^{3+} and Ca^{2+}

[Metal]/[Calmodulin] (mol/mol)	Metal	% α-Helix[a]
0	—	37
2	Ca^{2+}	40
4	Ca^{2+}	49
2	Al^{3+}	28
5	Al^{3+}	22

[a] calculated according to the method of Chen et al.[10]. These values represent the mean of at least three calmodulin preparations within 5% S.E.

This work was supported by US Department of Energy contract No. DE-AC01-76ER01338.

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(Received August 20, 1982; revised November 26, 1982)

CHAPTER IV

Organic Acids Prevent Aluminum-Induced Conformation Changes in Calmodulin

Vol. 119, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1984 Pages 376-381

ORGANIC ACIDS PREVENT ALUMINUM-INDUCED CONFORMATIONAL CHANGES IN CALMODULIN

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Received January 25, 1984

At a molar excess of 10:1 for [citrate]/[calmodulin], citrate can prevent aluminum binding to calmodulin when present in the protein solution in micromolar concentration, as determined by fluorescence and circular dichroism spectroscopy. In contrast, citrate is only partially effective in restoring calmodulin to its native structure once the aluminum-calmodulin complex (3:1) is formed, as measured by the α-helix content of the protein. Considering the magnitude of the stability constant of the citrate-aluminum chelate, citrate and perhaps other carboxylic acids may protect calmodulin, and thus cells, from toxic aluminum ions.

Calmodulin (CaM) has been shown to be a possible biochemical lesion for aluminum in the cell (1-4). In these studies it was demonstrated that the binding of aluminum ions to the protein results in both a loss of α-helical content and regulatory function of CaM. Furthermore, the ATP-associated formation of the transmembrane potential in plasma membrane-enriched barley root fractions was stimulated about twofold by bovine brain CaM. However, in the presence of micromolar concentrations of aluminum, the CaM-dependent potential formation was dissipated (3). Since CaM fulfills a pivotal regulatory role within the cell (5), protective mechanisms should exist that prevent the inactivation of CaM by toxic metals entering the cell. Putative protective mechanisms are those involving naturally occurring organic acids which have been implicated in affording protection to aluminum-tolerant plants (6,7). One such organic acid is citrate whose aluminum chelate (1:1) has a stability constant of about 10^8 (8). In addition, citrate has also been implicated in the transmembrane transport of metals in membrane vesicles of Bacillus subtilis (9) and in iron transport in higher plants (10). Moreover, aluminum hydroxy citrate complexes have been identified in the heartwood of the

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tree Adinandra brasii, found growing on aluminum toxic soil in New Guinea (11).

Employing spectroscopic methods we show in this communication that application of citrate can partially restore the aluminum-induced loss of structure in CaM, or, if added prior to aluminum addition, protect the regulatory protein from undergoing a loss of α-helix content. Citrate can therefore function in both a protective and a restorative role with respect to the regulatory protein (12). A high organic acid content may provide cells with a resistance mechanism against the deleterious effects of aluminum ions.

MATERIALS AND METHODS

Bovine brain acetone powder was purchased from Sigma Chemical Co. (St. Louis, MO). Calmodulin was isolated via phenothiazine affinity chromatography as previously described (2,13).

Fluorescence measurements were performed on a spectrofluorimeter from SLM Instruments (Urbana, IL), model 4000. The experiments were carried out at room temperature. The sodium salt of the fluorescent probe 8-anilino-1-naphthalene sulfonic acid (ANS) was purchased from K&K Laboratories (Plainview, NY). Excitation and emission wavelengths were 360 and 490 nm, respectively, and slit widths were set at 8 nm. The fluorescence data are the average of at least two determinations per point, and signal averaging was used so that each data point is a composite of a minimum of 50 scans.

Circular dichroic spectra were recorded on a Jasco spectropolarimeter, model ORD/UV/CD-5, modified by Sproul Scientific Instruments (Boulder Creek, CA). The experiments were carried out at room temperature. The percent α-helix content was calculated according to the procedure of Greenfield and Fasman (14) from the relation : percent α-helix = $-([\theta]_{222} + 2340)/303$. The mean residue ellipticity at 222 nm, $[\theta]_{222}$, is obtained from the measured value θ_{obs} at 222 nm and the relation $[\theta] = \theta_{obs}M/100\ell c$ (15), where M is the mean residue weight of calmodulin with a value of 117 (15); ℓ is the pathlength of the cuvette in cm, c is the protein concentration in g/cm^3.

All cuvettes and glassware were acid washed in concentrated nitric acid and rinsed with glass-distilled deionized water; plastic ware was treated with Chelex-100 (Bio-Rad Lab., Richmond, CA). Buffers were decontaminated of residual metals by passage through Chelex-100 columns.

Salts of $AlCl_3 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$ and citric acid were obtained from Mallinckrodt (St. Louis, MO). Tris was purchased from Sigma Chemical Co. (St. Louis, MO), and PIPES was obtained from Calbiochem (San Diego, CA). All other chemicals used were of the highest quality available.

All experiments were carried out at pH 6.5 since this value is representative of the cytoplasmic pH in plant roots (16).

RESULTS

Titration of bovine brain CaM with aluminum results in a substantial increase in ANS fluorescence intensity (Fig. 1). This increase can be attributed to the enhanced partitioning of the probe into the protein's nonpolar regions relative to a polar environment (17). At a molar ratio of

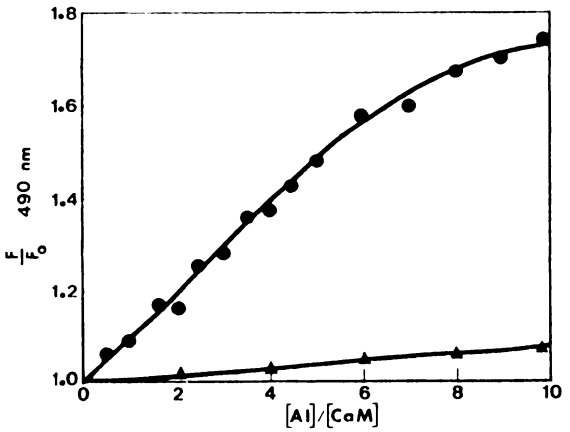

Figure 1. Aluminum-induced hydrophobic surface exposure of bovine brain calmodulin, in the absence (●) and in the presence of 100 μM citrate (▲). The concentration of calmodulin was 10 μM in 10 mM PIPES buffer, pH 6.5. The fluorescent, hydrophobic probe 8-anilino-1-naphthalene sulfonate (ANS) was added to give a final concentration of 2 μM. Citrate was added prior to titration of the protein solution with aluminum (▲). The fluorescence intensity F_0 is that of ANS in the absence of aluminum ions.

10:1 for [citrate]/[CaM], preaddition of citrate to the CaM solution effectively prevents an increase in the aluminum-triggered ANS fluorescence intensity upon subsequent titration of CaM with aluminum (1:4). Moreover, upon formation of the aluminum-CaM complex, organic acids were able to partially reverse the aluminum-induced conformational change of the protein (Figs. 2,3). The efficacy of the reversal follows the sequence: citrate>oxalate>malate> tartrate. A 50 percent reduction in the aluminum-induced ANS fluorescence enhancement of calmodulin was obtained at a molar ratio of 3:1 for [citrate]/[aluminum]. Complete reversal of the aluminum-induced ANS fluorescence of CaM could not be achieved with any of the organic acids tested.

Circular dichroic spectra of CaM show an α-helix content of 32 percent for metal-free CaM, at pH 6.5, which is in agreement with values from earlier studies (1,2) (Fig. 3A). Upon titration with aluminum to a molar ratio of 4:1 for [aluminum]/[CaM], the helix content is reduced to 28 percent, which corresponds to a loss of about 12 percent of the protein's α-helix content (Fig. 3A). Subsequent titration with citrate to a molar ratio of 6:1 for [citrate]/[aluminum] restored the α-helix content of the protein to about 30 percent, corresponding to a loss of about 6 percent in helices relative to the

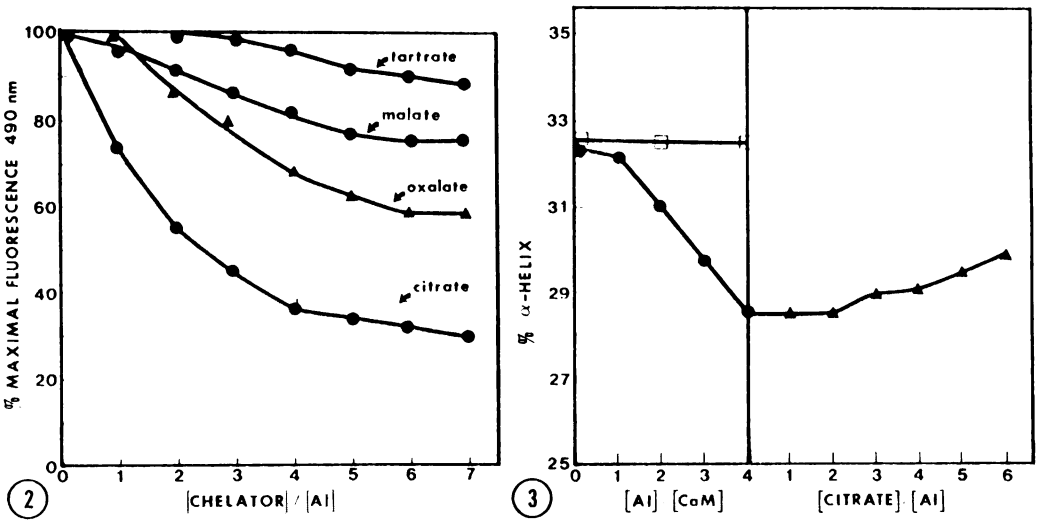

Figure 2. Decrease of aluminum-induced hydrophobic surface exposure of calmodulin, measured as maximal fluorescence intensity of 8-anilino-1-naphthalene sulfonate (ANS), following titration with organic acids as aluminum chelators. The concentration of calmodulin was 10 μM in 10 mM PIPES buffer, pH 6.5. ANS was added to give a final concentration of 2 μM. The 100 percent value of the ANS fluorescence intensity corresponds to that characteristic for a molar ratio of 3:1 for [aluminum]/[calmodulin].

Figure 3. Change in α-helix content of bovine brain calmodulin upon titration with aluminum in the presence of previously added 100 μM citrate (□), upon titration with aluminum in the absence of citrate (●), followed by titration with citrate (▲). Calmodulin was used at a concentration of 10 μM prepared in 10 mM Tris buffer, pH 6.5. The helix content was calculated according to the procedure by Greenfield and Fasman (14).

metal-free calmodulin (Fig. 3B). At a molar ratio of 10:1 for [citrate]/[calmodulin], preaddition of citrate to the protein solution prevented any detectable loss of α-helix by the protein upon titration with aluminum to a molar ratio of 4:1 for [aluminum]/[CaM] (Fig. 3A).

DISCUSSION

Our data show that a strong aluminum chelator such as citrate can prevent the binding of aluminum to CaM when the citrate is present in the protein solution prior to aluminum addition and in excess of aluminum ions. Once the aluminum-CaM complex is formed, citrate is only partially effective in restoring CaM to its native structure. At the micromolar concentrations used, citrate probably forms mononuclear chelates with aluminum (18,19,20). The stability constant for the mononuclear aluminum-citrate chelate falls within the range of about $10^{8}M^{-1}$ (8), which is about one order of magnitude

higher than that measured for the first mol of aluminum bound to CaM (2). Coordination of aluminum to citrate occurs through the two terminal carboxyl groups and the hydroxyl group according to recent ^{13}C-NMR studies (19). Reasons for the inability of citrate to fully restore CaM to its native state are: (a) upon binding of aluminum ions, CaM undergoes structural changes such that certain aluminum ions, bound to the protein, become inaccessible to citrate chelation; (b) binding of aluminum shifts the equilibrium between helical and coiled conformations of CaM as a result of metal-induced breakage of preexisting intramolecular hydrogen bonds of the protein (2), and citrate chelation of aluminum from CaM does not energetically favor the return of the helix-coil equilibrium to that of the protein's native state. These considerations are in accord with our results from circular dichroism studies indicating that the protein's α-helix content is not fully restored upon application of citrate to the aluminum-CaM complex. Further experiments are necessary to elucidate the structure of the aluminum-calmodulin complex in the presence and absence of aluminum-chelators.

Concerning citrate's protective role, our physico-chemical findings are consistent with those derived from physiological experiments. For example, when grown hydroponically in a medium that was supplemented with aluminum hydroxide and citrate, corn plants developed normally. However, corn plants displayed symptoms typical for aluminum toxicity when grown in the presence of aluminum with citrate absent (21). Measurements of intracellular citrate concentrations in soybean plants showed that citrate concentrations in xylem exudate approached 1 mM (10). Information on calmodulin concentrations in plant cells is lacking, however, erythrocyte data (22) indicate concentrations in the micromolar range. Consequently, our conclusion that a tenfold excess of citrate over calmodulin protects the protein from aluminum lesions appears to be a biologically relevant value.

ACKNOWLEDGEMENT

This project was supported in part by BRSG Grant #2-S07 RR07049-15 awarded by the Biomedical Research Grant Program, Division of Research Resources, National Institutes of Health.

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CHAPTER V

Citrate Chelation As A Potential Mechanism Against Aluminum Toxicity In Cells: The Role Of Calodulin

Citrate chelation as a potential mechanism against aluminum toxicity in cells: the role of calmodulin

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Received March 29, 1985

Suhayda, C. G. & Haug, A. (1985) Citrate chelation as a potential mechanism against aluminum toxicity in cells: the role of calmodulin. *Can. J. Biochem. Cell Biol. 63*, 1167–1175

At a molar excess of [citrate]/[aluminum], this organic acid can protect calmodulin from aluminum binding if the metal is presented to the protein in stoichiometric micromolar quantities, as judged by fluorescence and circular dichroism spectroscopy. Similar citrate concentrations are also capable of fully restoring calmodulin's hydrophobic surface exposure to that of the native protein when calmodulin was initially damaged by aluminum binding. Fluoride anions are equally effective in restoring calmodulin's native structure as determined by fluorescence spectroscopy. Measurements of the kinetics of citrate-mediated aluminum removal also indicated that the metal ions are completely removed from calmodulin, consistent with results derived from atomic absorption experiments. On the other hand, results from circular dichroism studies indicated that citrate-mediated aluminum removal from calmodulin can only partially restore the α-helix content to that originally present in apocalmodulin or in calcium–calmodulin, dependent upon the absence or presence of calcium ions. The results that chelators like citrate can protect calmodulin from aluminum injury may provide a conceptual understanding of physiological observations regarding aluminum-tolerant plant species which are generally rich in certain organic acids.

Suhayda, C. G. & Haug, A. (1985) Citrate chelation as a potential mechanism against aluminum toxicity in cells: the role of calmodulin. *Can. J. Biochem. Cell Biol. 63*, 1167–1175

La spectroscopie de fluorescence et le dichroïsme circulaire permettent de montrer qu'en présence d'un excès molaire de [citrate]/[aluminium], cet acide organique peut protéger la calmoduline contre la liaison à l'aluminium si le métal est présenté à la protéine en quantités micromolaires stoechiométriques. Des concentrations similaires de citrate sont aussi capables de restaurer complètement l'exposition superficielle hydrophobe de la calmoduline à celle de la protéine native quand la calmoduline est d'abord endommagée par la liaison à l'aluminium. Comme le montre la spectroscopie de fluorescence, les anions fluorure sont également efficaces pour restaurer la structure originale de la calmoduline. Les mesures de la cinétique de l'enlèvement de l'aluminium par l'intermédiaire du citrate indiquent aussi que les ions métalliques sont complètement disparus de la calmoduline, ce qui confirme les résultats obtenus lors des expériences d'absorption atomique. Par ailleurs, les études du dichroïsme circulaire montrent que l'enlèvement de l'aluminium de la calmoduline par le citrate ne restaure que partiellement la teneur α-hélocoïdale originalement présente dans l'apocalmoduline ou dans la calcium–calmoduline, selon que les ions calcium sont présents ou absents. Ces résultats voulant que des chélateurs comme la citrate puissent protéger la calmoduline contre les dommages causés par l'aluminium fourniraient une explication conceptuelle des observations physiologiques concernant les espèces végétales tolérant l'aluminium qui sont généralement riches en certains acides organiques.

[Traduit par la revue]

Introduction

Currently there exists no unifying theory regarding the primary biochemical lesion produced by toxic metals. Many diverse metabolic and structural changes associated with metal toxicity in cells probably result from a cascading process which originates from a primary lesion, e.g., the binding of the respective metal to a key regulatory protein like calmodulin.

The importance of the multifunctional protein calmodulin in calcium regulation is widely accepted, although the mechanistic details of its regulatory effects in cells are still rather unclear (1, 2). Found in all eukaryotes, this versatile protein harbors four calcium-binding domains with affinities in the micromolar range (3). Upon the binding of calcium to calmodulin, architectural and functional events take place that permit

ABBREVIATIONS: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; ANS, 8-anilino-1-naphthalene; Mes, 2-(*N*-morpholino)ethanesulfonic acid; CD, circular dichroism; Me, metal; CaM, calmodulin; Al, aluminum.

Printed in Canada / Imprimé au Canada

calmodulin to become an activator for a variety of target proteins.

Since calcium induces specific structural rearrangements, the protein's conformation and thus its activity may be drastically altered with the binding of toxic metals to the regulatory protein. One such metal is aluminum which has been shown to produce pronounced alterations in calmodulin's structure and function. For instance, addition of stoichiometric quantities of solvated aluminum species to micromolar calmodulin solutions causes the protein's α-helix content to decrease concomitantly with an enhanced hydrophobic surface exposure (4, 5). These aluminum-induced structural changes are reflected in biochemical malfunctions: e.g., the calcium-calmodulin-dependent 3′,5′-cyclic-nucleotide phosphodiesterase is inhibited (4) and the calmodulin-dependent ATPase activity, partially responsible for the maintenance of the transmembrane potential in barley root plasma membranes, is impaired (6). Although aluminum ions (7), like calcium ions (8), strongly prefer oxygen-containing ligands, there exists evidence which suggests that aluminum ions do not displace calcium ions from their specific binding sites (9). Possible binding sites for solvated aluminum species are carboxylic ligands of the acidic protein. The binding affinity for the first aluminum bound to the protein has been estimated to be about one order of magnitude stronger than that known for calcium (4). Contrary to the aluminum ion, the trivalent terbium ion can displace calcium from its specific sites while maintaining the functional integrity of calmodulin (10).

These aluminum-induced changes in calmodulin may constitute a molecular basis of aluminum toxicity. This broadly defined syndrome occurs in man where aluminum has been implicated in neurological disorders and in osteomalacia-type diseases (11). In plants, aluminum toxicity has been recognized as a serious global problem since vast areas of the world suffer from soil acidity which is a prerequisite for mobilization of aluminum in soil (12). Since calmodulin has been highly conserved during evolution and because this multifunctional protein fulfills a crucial regulatory role for many cellular functions, the hypothesis was advanced (13) that protective mechanisms should exist that prevent the inactivation by toxic aluminum ions which entered the cell. This notion is supported by findings that certain aluminum-tolerant plant species are rich in aluminum-chelating compounds such as organic acids (12).

To provide further evidence in support of this notion, we focus in this article on citrate and demonstrate that this organic acid can protect calmodulin from the deleterious effects of aluminum. Furthermore, experiments are being conducted to show that calmodulin, already injured by aluminum, can be rather completely restored upon application of citrate. These results thus indicate that a high content of citrate or similarly effective chelators may provide cells with a resistance mechanism against aluminum injury. The data are obtained by applying spectroscopic and biochemical methods.

Materials and methods

Materials

Affi-Gel–phenothiazine, AG 1X-8, and Chelex-100 were obtained from Bio-Rad Labs (Richmond, CA). Bovine brain acetone powder, DEAE-Sephadex A-50, ATP, cGMP, catechol, EDTA, EGTA, Tris, bovine heart 3′,5′-cyclic-nucleotide phosphodiesterase (activator deficient), and 5′-nucleotidase were purchased from Sigma Chemical Co. (St. Louis, MO). Pipes was obtained from Calbiochem (San Diego, CA). The sodium salt of the fluorescent probe ANS was purchased from K & K Laboratories (Plainview, NY). $AlCl_3 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, and citric acid were obtained from Mallinckrodt (St. Louis, MO). The ammonium salt of [8-^{3}H]guanosine-3′,5′-cyclic phosphate was purchased from New England Nuclear Co. (Boston, MA). All other chemicals were of the highest quality available. All cuvettes and glassware were washed with concentrated nitric acid and rinsed with glass-distilled water, whereas plastic ware was treated with Chelex-100. Buffers were decontaminated of residual metals by passage through Chelex-100 columns.

Calmodulin purification

Bovine calmodulin was isolated via phenothiazine affinity chromatography as previously described (14). The purity of the isolated protein was enhanced when the following modifications were incorporated into the isolation procedure. Prior to phenothiazine affinity chromatography, the clarified solution obtained after centrifugation at 100 000 × *g* was dialyzed overnight against 10 m*M* Tris–HCl (pH 7.5), containing 200 m*M* KCl and 1 m*M* mercaptoethanol, and then loaded onto a DEAE-Sephadex A-50 column (7 × 1 cm) which had been equilibrated with the same buffer. The column was washed until the absorbance of the eluate at 280 nm, monitored by an LKB 8300 Uvicord II (LKB-Produkter AB, Bromma, Sweden), had reached base line. The column was then eluted with the same buffer containing 450 m*M* KCl and 1 m*M* $CaCl_2 \cdot 2H_2O$, and the fraction collected was dialyzed overnight against 20 m*M* Mes–NaOH (pH 7.0), 1 m*M* mercaptoethanol, 300 m*M* NaCl, and 5 m*M* $CaCl_2$. This fraction was applied to a 20-mL phenothiazine affinity column, which had been equilibrated with the 20 m*M* Mes–NaOH buffer containing 1 m*M* $CaCl_2$, and then washed with the buffer until the eluate reached base line as monitored at 280 nm. A second wash with 20 m*M* Mes–NaOH buffer containing 500 m*M* NaCl and 1 m*M* $CaCl_2$ was applied. Calmodulin was eluted by washing the column with 20 m*M* Mes–NaOH buffer containing 500 m*M* NaCl and 10 m*M* EGTA. To the eluate $CaCl_2$ was added to give a final concentration of 15 m*M* and this fraction was dialyzed against 10 m*M* ammonium bicarbonate, distilled water, and distilled water containing Chelex-100 resin and finally electrodialyzed for 4 h at an applied current of 5–10 mA. The calmodulin prepared in such a manner was freeze-dried and stored desiccated below 0°C. Analysis of the calmodulin preparation on 15% polyacrylamide gels followed by Coomassie blue staining revealed a single band migrating at an apparent molecular weight of about 17 000, this result being

consistent when up to 90 g protein was loaded per gel lane. The purity of this protein was further documented by the absence of fluorescence emission upon excitation at 295 nm which represents the long wavelength region of the tryptophan absorption band (15). Tryptophanyl residues are constituents of many proteins as opposed to calmodulin which lacks such amino acid residues (2). Enzyme assays with 3′,5′-cyclic-nucleotide phosphodiesterase showed that the calmodulin preparation had biological activity in that it could stimulate the enzyme. As to the metal content of the purified calmodulin, atomic absorption experiments indicated that the [aluminum]/[calmodulin] molar ratio was 0.15 and that of [calcium]/[calmodulin] was 0.6, respectively. Calmodulin concentrations were determined using a molar extinction coefficient of 3300 $M^{-1} \cdot cm^{-1}$, at 277 nm (16), and measuring the absorbance at this wavelength with a Gilford spectrophotometer, model 2400 (Oberlin, OH).

Fluorescence and circular dichroic measurements

Fluorescence and circular dichroic spectra were recorded as previously described (13). For ANS fluorescence measurements samples were prepared in 10 m*M* Pipes buffer (pH 6.5) with final calmodulin and ANS concentrations of 10 and 2 μ*M*, respectively, in a total volume of 2 mL. Calmodulin samples for circular dichroism studies were prepared in 2 mL of 10 m*M* Tris buffer (pH 6.5) to give a final protein concentration of 10 μ*M*. The mean residue ellipticity at 222 nm was calculated by the method of Wolff et al. (17), and employing this value the helix content of calmodulin was determined (18).

Stock solutions of $AlCl_3 \cdot 6H_2O$ and $CaCl_2 \cdot H_2O$ were usually prepared at a concentration of 20 m*M* in deionized, glass-distilled water. Titrations involved the consecutive addition of 1- to 2-μL aliquots of stock metal solution to 2 mL of the calmodulin sample to provide the desired final metal concentrations. Stock solutions of sodium citrate, ATP, KF, catechol, and cGMP were prepared at a concentration of 90 m*M* as described above and titrated into metal calmodulin solutions in a similar fashion. All stock solutions were freshly prepared at the beginning of each experiment. The volume change of the calmodulin sample, at the conclusion of a titration series, was less than 1% of the sample's initial volume.

All spectroscopic measurements were performed at pH 6.5. This value is representative to that found in the cytoplasm of plant cells (19) and in animal cells (20). We would also like to mention that, according to ^{27}Al-NMR experiments, aluminum ions do not interact with Tris buffer (21) used in our experiments. As to the fluorescent dye used, ANS is a hydrophobic surface probe used to monitor conformational changes in proteins. The quantum yield of ANS in water is very low, whereas binding of the dye to hydrophobic regions of proteins can enhance the quantum yield several 100-fold, with an accompanying blue shift of the fluorescence emission peak (22, 23).

Phosphodiesterase assay

Activator-deficient 3′,5′-cyclic-nucleotide phosphodiesterase from bovine heart was dissolved in 20 m*M* Tris (pH 7.0). The assay employed was a modification of the procedure described by Wolff et al. (17). The hydrolysis of cGMP was measured in 20 m*M* Tris (pH 7.0), unless noted otherwise; the total volume was 300 μL in 1.5-mL polypropylene tubes. The final concentrations of assay components were 25 μg 3′,5′-cyclic-nucleotide phosphodiesterase (2.8 U), 1 μg calmodulin (0.2 μ*M*), 25 μ*M* carrier-labelled cGMP, 50 μ*M* $CaCl_2$, and 0.5 U 5′-nucleotidase. Except for carrier-labelled cGMP and 5′-nucleotidase, all assay constituents were mixed in polypropylene tubes and incubated at 30°C for 10 min. The reaction was started by the addition of cGMP. Following a 15-min reaction time, the assay was stopped by boiling the samples for 1 min and subsequently the tubes were placed on ice for 5 min. The samples were reincubated for 10 min at 30°C, at which point 5′-nucleotidase was added and the reaction was allowed to proceed for 15 min. The reaction was stopped by the addition of a 0.6-mL aliquot of AG 1X-8 resin, diluted 1:2 (v/v) in 50% isopropanol. The vials were centrifuged to sediment the resin, and an aliquot of supernatant was withdrawn and analyzed on a TmAnalytic liquid scintillation counter, model 6895 (Elk Grove, IL), to determine the extent of cGMP hydrolysis. The data are the results of two independent enzyme assays with each data point representing four replications for each treatment.

Atomic absorption

This technique was applied to determine the amount of aluminum bound to calmodulin following dialysis against citrate. A 40 μ*M* solution of calmodulin was prepared in 10 m*M* Pipes buffer (pH 6.5) and to 5 mL of this solution aluminum was added to give a final concentration of 120 μ*M*. A dialysis membrane with a 10 000 molecular weight cutoff was exhaustively washed with glass-distilled deionized water, followed by treatment with Chelex-100. The dialysis bag was loaded with the aluminum–calmodulin solution and placed into a 150-mL nitric acid washed beaker containing 1.08 m*M* citrate. The container was kept in motion at a constant rate by a magnetic stirring bar. Samples (100 μL) were taken from within the dialysis bag and from the external solution. These samples were analyzed for aluminum with a Hitachi polarized Zeeman atomic absorption spectrophotometer, model 180-80 (Tokyo). Prepurified argon was used as a sheath gas for the graphite atomizer and high purity grade nitrogen gas was used as carrier gas. Pyrolytically coated graphite cuvettes were obtained from Hitachi. Injection volumes were 10 μL and spectrophotometric conditions were set as described (24).

Results

Citrate can prevent aluminum-induced structural changes in calmodulin

Since carboxyl groups are coordinating donors for aluminum chelation, organic acids are effective ligands for aluminum complexation (25). For example, citrate forms a stable complex with aluminum between pH 5 and 8.0, as determined by ^{27}Al-NMR experiments (25). When present in excess of aluminum ions, citrate can prevent metal-induced conformational changes in bovine calmodulin (13). At a [citrate]/[calmodulin] molar ratio of 10, addition of up to 10 aluminum ions per protein resulted in a change of ANS fluorescence of less than 5% at the highest metal concentration, compared with the fluorescence intensity prior to metal titration, at pH 6.5 (Fig. 1). In the absence of citrate, the respective aluminum-induced fluorescence intensity change

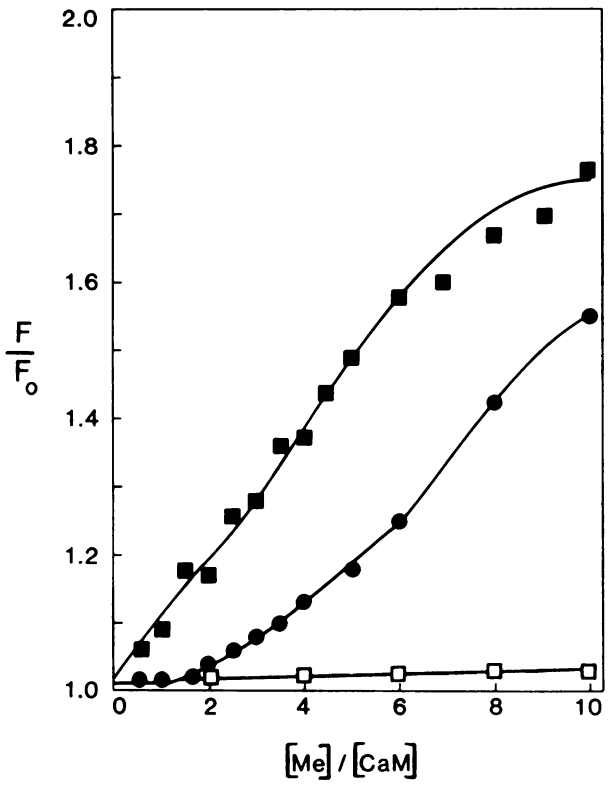

FIG. 1. Metal ion-induced hydrophobic surface changes in calmodulin, as measured by ANS fluorescence intensity emission at 490 nm, in the absence and presence of citrate. Aluminum ions were titrated onto calmodulin in the presence (□) and absence (■) of 100 μ*M* citrate. Similarly, calcium ions were titrated onto calmodulin in the absence (●) and presence (●) of 100 μ*M* citrate. The calmodulin concentration was 10 μ*M* in 10 m*M* Pipes buffer (pH 6.5); the final ANS concentration was 2 μ*M*. The abscissa indicates molar ratios of [metal]/[calmodulin]. F_0 represents the fluorescence intensity of the metal-free ANS-calmodulin complex and *F* represents the fluorescence intensity of the metal–calmodulin–ANS complex, formed upon addition of metal ions.

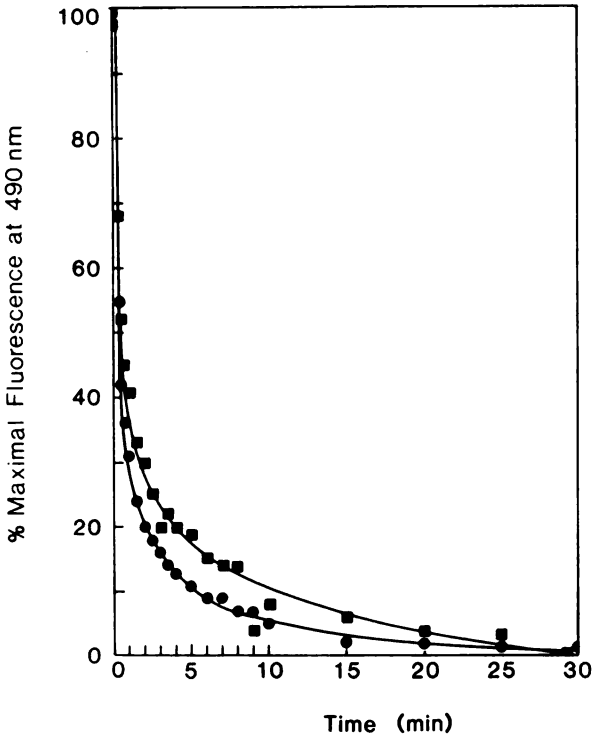

FIG. 2. Kinetics of citrate-mediated aluminum removal from calmodulin, in the absence (■) and presence of calcium (●). ANS fluorescence intensity was measured in 10 m*M* Pipes buffer (pH 6.5) containing 10 μ*M* calmodulin and 2 μ*M* ANS. The 100% fluorescence intensity value of the aluminum–calmodulin–ANS complex (■), at time zero, represents that at an [aluminum]/[calmodulin] molar ratio of 3, and the 0% fluorescence intensity value represents that of the metal-free calmodulin–ANS complex. Aluminum titrated onto the calcium–calmodulin–ANS complex, to an [aluminum]/[calmodulin] molar ratio of 3, in the presence of six calcium ions per protein (●) represents the 100% fluorescence intensity value, at time zero, while the zero intensity value corresponds to that of the calcium–calmodulin–ANS complex at a [calcium]/[calmodulin] molar ratio of 6. To start the citrate-mediated reaction, citrate was added to the sample to afford a final molar concentration of [citrate]/[aluminum] = 9, and after mixing the first reading was recorded after 15 s. ANS fluorescence intensity readings were taken over a 30-min period.

amounted to 80% at the highest aluminum concentration used. These data confirm our earlier results (13) which had indicated that citrate was effective in preventing aluminum-induced conformational changes in calmodulin.

In contrast, addition of 100 μ*M* citrate to 10 μ*M* calmodulin, prior to calcium addition, had no measurable effect on the ability of calcium to induce an enhancement of hydrophobic surface exposure of calmodulin. This increase was in fact identical to that observed in the absence of citrate, i.e., a fluorescence intensity increase of about 15% at a [calcium]/[calmodulin] molar ratio of 4.

These data therefore indicate the specificity of citrate as an effective aluminum ligand in the presence of calmodulin while apparently not interfering with the ability of calcium ions to associate with the regulatory protein.

Kinetics of aluminum removal from calmodulin by citrate

The time dependence of aluminum chelation by citrate following release of aluminum ions from calmodulin, in the absence and presence of calcium, was examined at different molar ratios of citrate per metal ion (Fig. 2). In the absence of calcium, measurements of

ANS fluorescence intensity were recorded over a 30-min period upon addition of citrate at various molar ratios of [citrate]/[aluminum] to the aluminum–calmodulin–ANS complex at time zero; prior to the addition of citrate, the molar ratio of [aluminum]/[protein] was 3. After 30 min, with a [citrate]/[aluminum] molar ratio of 9, the ANS fluorescence intensity value returned to that of the ANS–calmodulin complex at time zero, i.e., to that value characteristic for the apocalmodulin complex in the absence of aluminum. When lower molar ratios of competing citrate ligands were added to capture aluminum from calmodulin, the kinetics became more complicated since back reactions seemed to occur, as judged by the ANS fluorescence intensity values. When an excess of citrate ligands was present, these back reactions could be ignored and the kinetic system could be analyzed in terms of biphasic metal removal, following initial mixing of the reaction partners (about 15 s). Data for the kinetic process were evaluated in terms of a biphasic model after subtracting data representative of the asymptotic level (Fig. 2) from the measured curve. This asymptotic level represents the gradual return of the system to equilibrium upon removal of all three aluminum ions by citrate within a time of less than 8 min following citrate addition to the calmodulin solution. For times larger than 8 min, i.e., during the quasi steady-state process, characterized by the asymptotic level, it was assumed that all aluminum ions had been removed from calmodulin through citrate chelation, as judged by the ANS fluorescence intensity value and by the atomic absorption studies described below. The simplest interpretation for this type of kinetics is that two independent aluminum-binding sites on the protein have different rate constants for metal removal by the organic chelator. Since our experiments were performed at an [aluminum]/[protein] molar ratio of 3 and because the initial recording of ANS fluorescence could be taken about 15 s following citrate addition, the velocity of removal of the very first aluminum ion was probably so rapid as to exceed the recording capabilities of our instrument. For a [citrate]/[aluminum] molar ratio of 9, the kinetics of residual metal removal from calmodulin could thus be described as the sum of two exponential expressions. Following computer simulation of the kinetic curve, two rate constants were obtained, viz., $k_2 = 4.1 \pm 0.7\ \text{min}^{-1}$, and $k_3 = 0.6 \pm 0.08\ \text{min}^{-1}$, respectively. We also attempted to describe the observed kinetics in terms of a one-exponential function. However, in this case the fit of the computer-simulated curve to the measured data points was significantly worse than that generated by superimposing two exponential terms.

Since calmodulin, in the absence of calcium, seems to be a rather flexible molecule (26), as opposed to the more compact structure of calcium–calmodulin (4:1) (27), experiments were also conducted to measure the citrate-mediated removal of aluminum from calmodulin, in the presence of saturating calcium concentrations (Fig. 2). Under otherwise identical conditions as for the aluminum removal from apocalmodulin described above, the calcium concentration was adjusted to a [calcium]/[calmodulin] molar ratio of 6. As above, the best fit for the observed kinetics was obtained by assuming a biphasic removal with rate constants of $k_{2+} = 5.5 \pm 0.5\ \text{min}^{-1}$ and $k_{3+} = 0.5 \pm 0.04\ \text{min}^{-1}$, respectively. This means that the rate of removal of the second aluminum ion, k_{2+}, is accelerated by about 25% compared with the corresponding rate constant of aluminum removal from calmodulin, in the absence of calcium. Apparently the respective aluminum-binding sites on calmodulin, either in the absence or presence of calcium, are different, which in turn results in a different accessibility to the chelator. Moreover, in the presence of calcium, the asymptotic level defined above is representative of the fluorescence emission of the calcium–calmodulin complex rather than that of the apoprotein.

For the same [citrate]/[aluminum] molar ratio of 9, citrate is also effective in removing the metal from the protein according to dialysis experiments, performed in the absence of calcium. A solution of aluminum–calmodulin (3:1) was placed in a dialysis bag and dialyzed against an external citrate solution so that the [citrate]/[aluminum] molar ratio was 9. By taking samples from inside the bag, the molar ratio of aluminum per protein was monitored by atomic absorption spectroscopy. After 17 h of dialysis, this molar ratio had decreased to 0.32 from an initial value of 3, indicating a 90% reduction in metal content.

CD studies of aluminum ion interactions with calmodulin

At pH 6.5, the metal-free α-helix content was found to be 32%, and at pH 7.4, it was found to be 28%, a value consistent with data reported in the literature (17). The addition of 4 mol aluminum · mol calmodulin^{-1} resulted in a decrease of the helix content from 32 to 27.5%, the SD of the measurement being about 10%.

Addition of aluminum to a calmodulin solution containing citrate at a [citrate]/[aluminum] molar ratio of 10 demonstrated the protective effect of citrate, with respect to prevention of the loss in helix content when the metal was titrated into the solution to an [aluminum]/[calmodulin] molar ratio of 3, at pH 6.5. In agreement with our previous findings (13), these experiments document further the specificity of citrate for aluminum, because the application of calcium to apocalmodulin (4:1) produced the same helix content as that observed when calcium was titrated onto metal-free calmodulin.

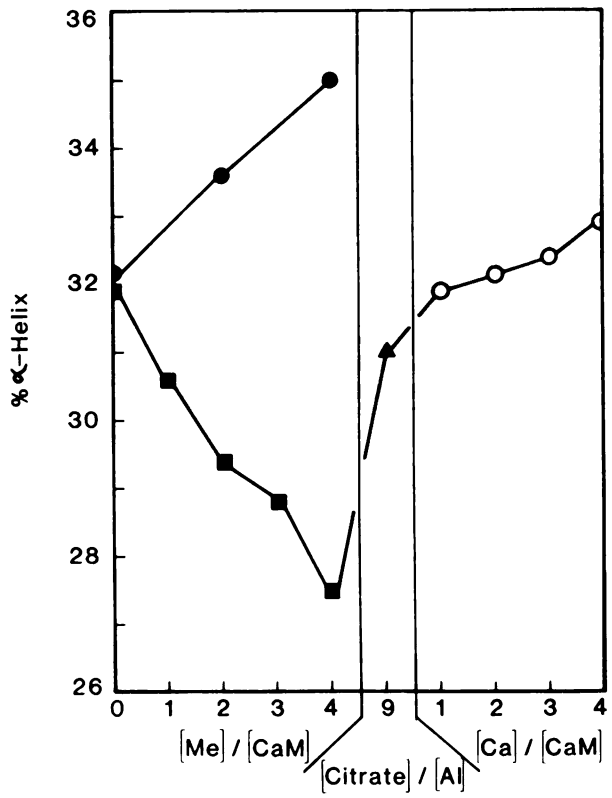

FIG. 3. Citrate partially restores the helix content of calcium–calmodulin following treatment with aluminum. Calcium added to 10 μ*M* calmodulin in 10 m*M* Tris buffer (pH 6.5) to a [calcium]/[calmodulin] molar ratio of 4 (●); addition of aluminum to an [aluminum]/[calmodulin] molar ratio of 4 (■); citrate added in excess of aluminum at a molar ratio of 9 (▲); titration of this citrate-treated calmodulin with calcium to a [calcium]/[calmodulin] molar ratio of 4 (○). The SD of these measurements was 10%.

As to restoration of calmodulin, upon application of aluminum ions at an [aluminum]/[calmodulin] molar ratio of 4, the helix content decreased. If citrate is applied subsequently at a [citrate]/[aluminum] molar ratio of 9, the helical content was only partially restored, after taking into account the kinetics of citrate-mediated aluminum removal discussed above. Addition of calcium, at a [calcium]/[calmodulin] molar ratio of 4, further increased the α-helix content, but not to that of calcium–calmodulin when calmodulin was titrated directly with calcium at the same molar ratio (Fig. 3).

Control experiments indicated that the presence of excess amounts of citrate in a calmodulin solution do not interfere with the binding of calcium to the protein, resulting in a gain in helix content. For example, when citrate was added to a calmodulin solution prior to loading calcium, at a [citrate]/[calmodulin] molar ratio of 10, the result was an overlapping curve identical to that obtained upon calcium titration in the absence of citrate. Similarly, within error of measurement, the α-helix content of calcium–calmodulin (4:1) remained unchanged upon titration of the sample with citrate at a [citrate]/[protein] molar ratio of 10.

TABLE 1. Chelator-mediated removal of aluminum from calmodulin

Chelator	Percentage
Citrate	100
NaF	100
ATP	75
Catechol	50
cGMP	0

NOTE: Prior to chelator addition, the maximal fluorescence intensity of 100% corresponded to that of the [aluminum]/[calmodulin] = 3 complex, in the presence of ANS. The values represent the percentage in reduction (error about 5%) of the initial fluorescence intensity value, 30 min after chelator addition.

Effect of other chelators on calmodulin injured by aluminum

Besides citrate, several other chelators were tested as to their efficiency in reducing the aluminum-induced lesion on calmodulin, as judged by ANS fluorescence intensity measurements (Table 1). For these experiments the chelator was applied at a [chelator]/[aluminum] molar ratio of 9 and the ANS fluorescence intensity was measured at 30 min following addition of the respective chelator. Similar to citrate, the fluoride anion is able to completely reduce the aluminum-induced ANS fluorescence of calmodulin to that associated with the ANS–apocalmodulin complex. ATP and catechol diminish the aluminum-induced ANS fluorescence intensity of calmodulin by 75 and 55%, respectively. The monophosphate cGMP was totally ineffective as an aluminum chelator when added to the aluminum–calmodulin complex. These findings on aluminum binding to mono- and tri-phosphates of nucleotides are in general agreement with studies on metal binding to nucleotides (28). Chelate formation of metals seems to occur with the purine ring and the beta- and gamma-phosphates involving the primary hydration shell of the metal, while binding to monophosphates involves the secondary hydration shell. Furthermore, the constrained structure of cGMP is probably even less favorable towards aluminum binding to the nucleotide.

Citrate protects calmodulin phosphodiesterase from aluminum injury

Aluminum has been shown to inhibit calmodulin stimulation of the calcium-dependent 3′,5′-cyclic-nu-

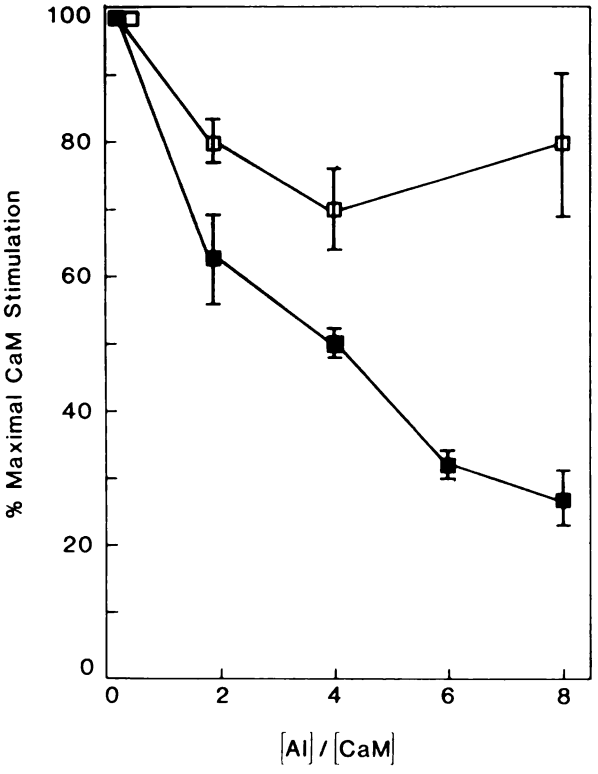

FIG. 4. Inhibition of calcium- and calmodulin-dependent 3',5'-cyclic-nucleotide phosphodiesterase activity by aluminum (■) and partial reversal of the inhibition by the addition of equimolar citrate concentrations (□). Citrate was applied in equimolar ratios to aluminum. On the abscissa molar ratios are listed. The bars represent the SD of each treatment. Stimulation of 100 percent corresponds to 0.18 nmol cGMP hydrolyzed·mL^{-1}·min^{-1}, whereas the basal activity was 0.14 nmol cGMP hydrolyzed·mL^{-1}·min^{-1}.

cleotide phosphodiesterase activity (4). The inhibitory effect of aluminum is the result of the metal's interaction with calmodulin rather than the enzyme, since aluminum concentrations employed in this assay had no measurable effect on the enzyme's basal activity. When assayed at pH 7.0, aluminum inhibited the calcium–calmodulin dependent phosphodiesterase activity by 50% at an [aluminum]/[calmodulin] molar ratio of 4 (Fig. 4). Prior addition of citrate to equimolar aluminum concentrations in the assay system resulted in inhibition of this enzyme system by only 20% approximately, at all aluminum concentrations tested. The addition of equimolar quantities of aluminum per citrate to the enzyme had no influence on the basal activity of the enzyme. However, in the absence of aluminum, addition of citrate stimulated the enzymatic activity. Since any excess of citrate over aluminum would stimulate the basal enzymatic activity, only equimolar concentrations were employed in the experiments described above. As far as equimolar concentrations are concerned, citrate plays a protective role in the aluminum-induced inhibition of the calcium–calmodulin dependent phosphodiesterase system (Fig. 4).

Atomic absorption measurements indicated that the application of excess citrate to an aluminum–calmodulin complex, with an initial [aluminum]/[calmodulin] molar ratio of 3, resulted in the reduction of this ratio to 0.3. Therefore, following citrate removal by dialysis, the metal-depleted protein was assayed as to its efficacy in stimulating phosphodiesterase activity. Stimulation of the enzyme by this citrate-treated calmodulin was identical to calmodulin which had not been treated with aluminum and citrate.

As to the citrate-induced stimulation of basal phosphodiesterase activity, even in the absence of aluminum, this stimulation persisted after extensive dialysis of the enzyme against Chelex-100. This observation seems to indicate that the citrate-related stimulation does not result from citrate-mediated withdrawal of aluminum ions from the possibly metal-contaminated enzymatic protein. Moreover, citrate-mediated removal of metals chelated to cGMP, the enzyme's substrate, can be ignored because metal binding to monophosphates is very low, as opposed to triphosphates (29). Thus, further experiments are necessary to clarify the citrate-induced stimulation of phosphodiesterase activity.

Discussion

The results presented in this report indicate that citric acid can protect calmodulin from aluminum injury, when citrate is present in the calmodulin solution and in excess of aluminum ions. At the micromolar aluminum concentrations used, mononuclear complexes of aluminum–citrate are formed (30). Aluminum is probably coordinated through citrate's two terminal carboxyl groups and the central hydroxyl group, as demonstrated by ^{13}C-NMR experiments (30). This mononuclear aluminum–citrate chelate has a stability constant which falls within the range of about 10^8 M^{-1} (31), i.e., one order of magnitude stronger than that estimated for the binding of the first aluminum ion to calmodulin (4). However, dimeric citrate–aluminum complexes (2:1) are known to exist when excess ligand is added to aluminum solutions in the millimolar concentration range (32). Evidently the presence of citrate does not interfere with the interaction of calcium and calmodulin. These data are in accord with stability constants for calcium binding to carboxylic acids (8) which are several orders of magnitude lower than the micromolar affinity constants reported for calcium binding to the protein (2, 3).

Our results derived from ANS fluorescence measurements show that application of excess citrate can apparently rather completely restore the hydrophobic

surface domains of the protein typical to those of apocalmodulin and calcium–calmodulin, dependent upon the absence or presence of calcium ions. On the other hand, restoration in terms of helical content appears to remain somewhat incomplete, as judged by CD measurements. This net loss of helix content was evident, irrespective of the presence or absence of saturating calcium concentrations. Apparently the aluminum-produced, profound perturbations in the protein's secondary structure also lead to a rearrangement of water molecules, because the protein's conformation is highly dependent upon water association (33). These rearranged water molecules may compete for hydrogen-bonding sites on calmodulin or may change the solvation in polar regions, when the protein refolds following citrate-mediated metal removal.

The citrate-mediated release of aluminum from calmodulin, in the presence or absence of calcium, proceeds in a time-dependent manner, requiring minutes to reach completion. The roughly 10-fold difference in the respective rate constants is statistically significant. The rate constants k_2 and k_3 correspond to half-lives of about 10 and 70 s, respectively. Similar time constants, varying between 10 and about 100 s, have been found in kinetic studies concerning folding reactions in ribonuclease (34), a protein with a molecular weight similar to that of calmodulin. As to the mechanism of citrate-mediated aluminum removal, possibly a conformational change of the protein can be the rate-limiting step. The lack of ready reversibility of the aluminum–calmodulin complex upon application of citrate presumably results from the profound denaturation initially produced by aluminum in the protein (4). The rate-limiting step might also be the dissociation of a mixed-ligand intermediate involving a ternary complex of citrate and aluminum–calmodulin. Such an intermediate has been identified spectroscopically in the aerobactin-mediated removal of iron transferrin, the serum iron transport protein (35). It appears worth noting that aerobactin is a bacterial iron transport carrier which belongs to the hydroxamic acid – citrate family of siderophores (35).

As to aluminum removal by chelators other than citrate, the results seem to indicate that the value of the stability constant approximately parallels the rate and extent of aluminum removal from the protein. This is illustrated by catechol whose stability constant with aluminum was estimated to be $8 \times 10^7\ M^{-1}$, at pH 6.5, as compared with a value of about $5 \times 10^8\ M^{-1}$ for aluminum–citrate (31). Presumably steric factors arising within the protein and the molecular size and charge of the chelator also play a role in the access of the ligand to buried aluminum sites. This is further documented by our findings that the capture of aluminum by citrate is diminished in aluminum–calmodulin, in the absence of calcium. Although on a time scale several orders of magnitude different from ours, access of quenching molecules to buried fluorophores in proteins has been found to be under partial control of the conformational fluctuations of the protein (36, 37).

What about the physiological significance of our findings with respect to metal-sequestering molecules produced by cells in response to toxic metals? Our physicochemical findings that certain chelators, especially citrate and fluoride, can protect calmodulin from aluminum injury may provide a conceptual understanding of a variety of physiological observations regarding aluminum-tolerant plant species. This is illustrated by the findings that in the roots of aluminum-tolerant Sorghum cultivars the organic acid fraction is enhanced compared with that in sensitive cultivars (38). Furthermore, maize plants showed symptoms typical for aluminum toxicity when grown in the presence of aluminum, with citrate absent (39). Besides organic acids, polyphenols in tea plants have been implicated as protectants against aluminum toxicity (12). Finally, addition of NaF to the growth medium clearly alleviated the aluminum-induced repression of tea pollen tube growth (40). Extracellularly, millimolar citrate concentrations have been found in xylem exudates of soybean plant (41), whereas intracellularly, the estimated citrate concentration of a plant cell is 0.1–1.0 mM for the mitochondria and cytoplasm and 0.5–8.0 mM for the vacuole (42). Our conclusion that a 10-fold excess of citrate over calmodulin prevents aluminum from injuring the protein seems to be a physiologically relevant value. In addition, the concentration of calmodulin in spinach was estimated to be approximately 1 μM from available data (43). Moreover, the micromolar aluminum concentrations employed, at pH 6.5, seem to be reasonable values since typical acidic soils contain between 30 and 100 μM mobile aluminum ions (44).

The specificity of the aluminum–calmodulin interaction in underscored by recent studies regarding heavy metal inactivation of calmodulin. For example, about 5- to 10-fold more divalent heavy metals are required to reduce calmodulin- and calcium-dependent phosphodiesterase activity by 50% (45), as compared with aluminum (4). The observed inactivation of calmodulin by heavy metals apparently correlates with their respective toxicity in mice (45).

In summary, certain organic acids may chelate aluminum and thus mediate the subsequent cellular detoxification of the metal, without interfering with the calcium regulation of calmodulin. The formation of the metal chelate prevents aluminum from inducing conformational and functional changes in calmodulin. In addition, the level of certain organic acids in cells may be a desirable genetic trait for the selection of aluminum-tolerant plant cultivars.

Acknowledgements

We thank Dr. J. Speck, Biochemistry Department,

and Dr. K. Poff, Plant Research Laboratory, for the use of the CD instrument and SLM spectrofluorimeter, respectively. We also wish to thank Don Versteeg, Pesticide Research Center, for assistance with atomic absorption measurements. This work was supported by grant PCM-8314662 from the National Science Foundation and a gift from Pioneer Hi-Bred International.

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CHAPTER VI

Organic Acids Reduce Aluminum Toxicity In Maize Root Membranes

Introduction

Aluminum is the most abundant metal in the earth's crust and since vast areas of the world suffer from soil acidity, they suffer from aluminum toxicity as a consequence (Foy et al. 1978). The biochemical basis for the mechanism(s) of aluminum toxicity at the cellular level remains elusive despite a rather large body of data available regarding the metal's toxic effects in plants and animals at both the organismic and molecular levels (Foy et al. 1978, King et al. 1981, Haug, 1984). The root is the principal organ responsible for the uptake and transport of this metal to other plant organs. In corn roots, the primary site of aluminum uptake seems to involve cells on the periphery of the root cap where also mucilage is secreted (Bennet et al. 1985a). Macroscopic changes in root anatomy and morphology accompanying aluminum stress include a reduction in root length, suppression of lateral root formation and depressed root hair development, especially in aluminum-sensitive varieties (Fleming and Foy 1968).

The initial changes associated with aluminum stress in root tissue appear to be alterations in subcellular membranes and membrane-mediated processes. In Al-tolerant varieties of snap bean, aluminum stress leads to an increase in intracellular calcium concentrations, as opposed to reduced calcium levels in Al-sensitive varieties (Foy et al. 1972). In the presence of aluminum ions, sustained calcium absorption and transport was indicative of aluminum tolerance of wheat, barley and soybean varieties (Foy et al. 1969, Foy et al. 1967). Depolarizations of the membrane potential of root cells of red spruce (Picea rubens), and black willow (Salix nigra), resulted from the exposure of root tissue

to aluminum ions (Etherton et al. 1983). These changes in cellular electrogenic activity were observed immediately after application of aluminum ions. Similarly, in plasma membrane enriched fractions from barley roots, application of aluminum ions has been shown to dissipate the calmodulin (CaM) stimulated transmembrane potential, presumably a consequence of aluminum-induced conformational changes in the regulatory protein (Siegel and Haug 1983b). Ultrastructural studies on root cap cells of corn revealed that the initial subcellular response to aluminum stress is apparently the disruption of the migration of secretory vesicles from dictyosomes to the plasma membrane, followed by disruption and alterations of the dictyosome membrane (Bennet et al. 1985b).

The basis of aluminum tolerance in plants is not known although several hypotheses have been proposed. Certain Al-tolerant wheat varieties can raise the pH in the root zone which, in turn, reduced the accessibility of the metal in the soil solution and thus its uptake by the root (Foy et al. 1965). Aluminum has been found to accumulate at higher concentrations in subcellular fractions of Al-tolerant wheat varieties as compared with sensitive varieties and thus an "avoidance mechanism" of aluminum tolerance is apparently excluded (Niedziela and Aniol 1983). Chelation of aluminum ions by organic acids has been invoked as a means to detoxify these metal ions since plants can accumulate these aluminum chelates without apparent toxic effects (Jones 1961, Bartlett and Riego 1972, Foy et al. 1978, Jayman and Sivasubramanian 1975).

In this study we present information on quantitative and qualitative changes in the water-soluble organic acid content of roots of Al-tolerant and Al-sensitive corn hybrids when stressed with aluminum in the culture medium. We also examine potentially protective effects of organic acids on the plasma membrane's physical properties and activity of the Mg^{2+}-dependent ATPase isolated from corn root tissue when exposed to deleterious aluminum ions. Our data support the notion that organic acids, in particular malate and citrate, when present in corn roots at sufficient concentrations, can serve to reduce the impact of toxic aluminum ions on crucial cellular components and thus on physiological processes.

Abbreviations - Al, aluminum; EPR, electron paramagnetic resonance; ANS, 8-anilino-1-naphthalene sulfonate; Mes, morpholinoethane sulfonic acid; Pipes, 1,4-piperazinediethane sulfonic acid; EDTA, ethylenediaminetetraacetic acid.

Materials and Methods

Culture of corn (Zea mays)

An Al-tolerant corn cultivar, W64ANRHt, and an Al-susceptible corn cultivar, A632Ht, were obtained from the Agricultural Alumni Seed Improvement Association, Inc., of Romney, Indiana. Prior to being placed on germination trays, the corn seeds were allowed to soak in tap water for 4 to 5 h, surface sterilized in a 5% (v/v) sodium hypochlorite solution for 10 min, and washed extensively with tap and distilled water. The seeds were placed on rubber mesh germination trays (35 cm x 27 cm) and positioned on top of plastic trays (35 cm x 30 cm x 9 cm) which contained two air stones glued to the tray bottom to facilitate

aeration for the culture media. The trays were covered and the seeds were germinated and grown at 22°C. A 0.25 mM $CaSO_4$ solution, pH 6.5, was used as the germination and culture medium. Corn grown without aluminum supplementation was solely cultured in 0.25 mM $CaSO_4$ solution and the roots harvested 5 to 10 days after germination. Aluminum-stressed corn was cultured in 0.25 mM $CaSO_4$ for the first 7 days after germination and then supplemented with 150 µM aluminum solution ($AlCl_3 \cdot 6H_2O$) in addition to calcium at pH 5.0 for three additional days and the roots then harvested. The seedlings were provided with fresh culture medium daily and the trays were cleaned to minimize microbial growth.

Extraction and Analysis of Organic Acids

Corn roots were harvested by cutting with scissors and frozen in liquid nitrogen. The frozen tissue was placed into a lyophilization flask and dried on a lyophilizer; the dried tissue was stored at -40°C until analysis was performed. Organic acid extraction and derivatization was accomplished by application of a micromethod procedure useful for quantitating organic acids in as little as 0.1 g fresh weight of plant tissue (Stumpf and Burris 1979). The volatile derivatives of the extracted acids were prepared by dissolving the dried acid fraction in anhydrous pyridine followed by the addition of N,O-Bis-(trimethylsilyl)acetamide (Pierce, Rockford, IL) which resulted in the formmation of the trimethylsilyl derivatives of the organic acids after 10 min at room temperature. The silylated mixtures of organic acids were chromatographed on a Hewlett-Packard gas chromatograph, model 402, and the elution profiles recorded on a Hewlett-Packard integrator, model 3380A. A Sylon-CT (Pierce, Rockford, IL) treated glass column with dimensions 0.4 cm x 180

cm was packed with 15% SE-52 on Chromosorb W-HP, 80/100 mesh (Supelco, Bellefonte, PA), and used throughout the analysis. The temperature program applied was 130 to 260°C at 5°C/min after an initial program delay of 1 min. The injector and detector temperatures were 215°C and 270°C, respectively. The organic acids were identified by comparing the retention time and elution temperature of the unknowns with trimethylsilyl derivatives of standard organic acids.

Membrane Isolation

A plasma membrane enriched microsome fraction was isolated from the Al-susceptible corn cultivar, A632Ht. Seedlings were grown as described above, however, they were not treated with aluminum and the roots were harvested after 7 to 8 days. Plasma membranes were isolated according to published procedures (Leonard and Hotchkiss 1976, Caldwell and Haug 1980). Briefly, the roots were cut from the seedlings with scissors, rinsed with distilled water, blotted on paper towels, and then weighed. The roots were macerated with a razor blade for 1 to 2 min and placed in a chilled mortar with 3 volumes per gram fresh weight of cold homogenizing buffer, composed of 25 mM Tris-Mes, pH 7.7, 3 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride, and 0.25 M sucrose. The macerated tisue was ground with a pestle for 3 to 5 min, and then filtered through four layers of Miracloth (Calbiochem, San Diego, CA). The homogenate was centrifuged for 15 min at 12,000 g. The resulting supernatant was centrifuged for 30 min at 80,000 g. The tan pellet was removed and resuspended in 1 mM Tris-Mes buffer, pH 7.7, containing 0.25 M sucrose, using a chilled ground glass homogenizer. This crude microsome fraction was layered onto a 34%/40% (w/w) discontinuous

sucrose gradient prepared in 1 mM Tris-Mes buffer, pH 7.2, and then centrifuged for 2 h at 80,000 g. The plasma membrane enriched microsome fraction was removed from the 34%/40% interface of the sucrose gradient, diluted with 1 mM Tris-Mes, pH 7.2, buffer containing 0.25 M sucrose and centrifuged for 30 min at 80,000g. The resulting pellet was homogenized in the same buffer and frozen at -40°C. This plasma membrane fraction was used for enzyme assays and the EPR experiments. Membrane protein concentrations were determined with bovine serum albumin as a standard (Wang and Smith 1975). NADPH cytochrome C reductase activity was assayed according to published procedures (Hodges and Leonard 1974).

ATPase Assays

The Mg^{2+}-dependent ATPase activity was assayed according to published procedures (Leonard and Hotchkiss 1976). Tris-ATP was prepared by adding beads of Dowex 50W-X1 (H^+ form), 50 to 100 mesh resin, to a 60 mM ATP solution to afford a final pH of 2.0. The resin was removed by filtration and solid Tris base crystals added to give a final pH value of 6.5. Assays were performed in 30 mM Tris-Mes buffer, pH 6.5, in a total volume of 1 ml, and contained the following concentrations of each component: 3 mM Tris-ATP, 3 mM $MgSO_4 \cdot 7H_2O$, 50 mM KCl (when required), 20 µg membrane protein. The reaction was started by the addition of Tris-ATP to the preincubated enzyme mixture and allowed to proceed for 30 min at 22°C at which time it was stopped by the addition of 0.25 ml chilled 26.7% (w/w) trichloroacetic acid. The ATPase activity was linear for at least 45 min. The amount of inorganic phosphorus was measured colorimetrically (Rathbun and Betlach 1969).

Calmodulin Purification

Calmodulin was isolated from bovine brain acetone powder using phenothiazine affinity chromatography (Caldwell and Haug 1981a, Suhayda and Haug 1985).

EPR and Fluorescence Experiments

For EPR experiments the plasma membrane fraction was resuspended in 10 mM Pipes buffer, pH 6.5, to give a final membrane protein concentration of 1 mg/ml. Samples were prepared by adding the spin probe, 5-doxyl stearic acid (Molecular Probes, Junction City, OR), in hexane into a 1.5 ml polypropylene test tube, evaporating off the hexane, and then adding the diluted membrane to the tube. The membrane was mixed on a Vortex mixer to incorporate the spin label into the membrane. The concentration of the spin label was maintained at 6 nmol/mg membrane protein. 1 to 2 µl aliquots of freshly prepared stock solutions (1 to 50 mM) of $AlCl_3 \cdot 6H_2O$, or $CaCl_2 \cdot 2H_2O$, or sodium citrate were titrated into 100 µl samples of spin labelled membrane, resulting in final metal or citrate concentrations of 10 to 1000 µM.

EPR spectra were recorded on a Varian X-band spectrometer, model E-112, at power and modulation amplitude settings which were below those causing saturation or linewidth broadening. The hyperfine splitting parameter, $2T_{//}$, obtained from the spectra provides information on the mobility of the spin probe in the membrane microenvironment. An increase in the value of $2T_{//}$ is associated with motional restraints of the probe in its microenvironment (Griffith and Jost 1976).

Fluorescence spectra of the dye, 8-anilino-1-naphthalenesulfonic acid (ANS), associated with calmodulin were recorded as previously

described (Suhayda and Haug 1984). This dye is routinely used to monitor conformational changes in proteins. Binding of ANS to hydrophobic regions of proteins can result in a several hundred fold enhancement of the dye's fluorescence quantum yield and is accompanied by a blue shift in the emission peak compared with fluorescence characteristics in aqueous solutions (Stryer 1965, Slavik 1982). ANS fluorescence experiments with calmodulin were performed in 10 mM Pipes buffer, pH 6.5, with final calmodulin and ANS concentrations of 10 and 2 μM, respectively, in a total volume of 2 ml.

Materials

Tris and ATP were purchased from Sigma Chemical Co. (St. Louis, MO). Pipes was obtained from Calbiochem (San Diego, CA). Chelex-100 was obtained from Bio-Rad Labs (Richmond, CA). The sodium salt of the fluorescent probe ANS was purchased from K and K Laboratories (Plainview, NY). $AlCl_3 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, and citric acid were obtained from Mallinckrodt (St. Louis, MO). Succinate, fumarate, malonate, malate, trans-aconitate and quinate were purchased from Fluka Chemical Co. (Hauppauge, NY). All other chemicals were of the highest quality available. Cuvettes and glassware were nitric acid washed and rinsed with glass-distilled water; plastic ware was treated with Chelex-100 to remove contaminating metals. Buffer solutions were passed over Chelex-100 columns to remove any residual metals.

Results

Organic acid content of corn roots in response to aluminum stress

Under our conditions the silyl ester derivatives of the organic acids, with the exception of trans-aconitate, were stable for several hours at room temperature. In agreement with a previous report (Stumpf and Burris 1979) the volatile trans-aconitate derivatives were unstable at room temperature showing maximal peak response 10 min after mixing with BSA and having on the average a 34% reduction in peak response 45 min after mixing while showing no peak 3 hr after BSA addition. The rapid degradation of silyl ester derivatives of trans-aconitate in conjunction with longer reaction times (30 min) of sample with BSA (Popp and Kinzel 1981) may account for the 3- to 5-fold higher levels of trans-aconitate we observed in corn root tissue in comparison to reported values for this organic acid in sorghum root tissue (Guerrier 1982, Cambraia et al. 1983) apart from naturally occurring differences between the two plant species.

Analysis of the water soluble organic acids of the Al-sensitive cultivar, A632 and the Al-tolerant cultivar, W64 revealed that the predominant organic acids in both Al-stressed and non-stressed root tissue were trans-aconitate and malate. As shown in Table 1, non Al-stressed A632 contained an average of 42.8 µmol/g dry wt trans-aconitate and 13.8 µmol/g dry wt malate, accounting for 75% of the total water soluble organic acid content of these roots. Analysis of non Al-stressed W64 roots showed 45.6 µmol/g dry wt trans-aconitate and 17.4 µmol/g dry wt malate in this tissue representing 80% of the total water soluble organic acid content. Following trans-aconitate and malate in decreasing

Table 1. Organic acid content of roots of Al-stressed and non-stressed corn seedlings.

Organic acid	A632 non-stressed	A632 Al-stressed	W64 non-stressed	W64 Al-stressed
Succinate	11.1	4.0	13.2	4.3
Fumarate	<1.0	<1.0	<1.0	<1.0
Malonate	<1.0	<1.0	<1.0	<1.0
Malate	13.8	7.5	17.4	11.6
t-Aconitate	42.8	13.0	45.6	24.1
Citrate	4.7	0.8	1.1	1.3

Al-stressed maize was cultured in 0.25 mM $CaSO_4$ for the first seven days after germination, then supplemented with 150 µM Al solution, in addition to calcium, at pH 5.0 for three additional days, and the roots then harvested. The values expressed as µmol/g·dry wt represent the average of at least two determinations for each treatment. A632 is the Al-sensitive maize hybrid and W64 is the Al-tolerant hybrid. SD about 7%.

concentration in non Al-stressed root tissue of A632 and W64, respectively, was succinate (11.1 and 13.2 µmol/g dry wt), citrate (4.7 and 1.1 µmol/g dry wt) and fumarate, malonate and quinate; the final three present at levels below 1.0 µmol/g dry wt. The net effect of Al-stress on both A632 and W64 was the overall reduction in the organic acid content of root tissue by 63% and 45%, respectively. Comparison of Al-stressed A632 roots to non Al-stresed roots showed that the concentrations of trans-aconitate and malate were reduced by 70% and 46%, respectively, in contrast to W64 where 47% and 33% reductions in the levels of these two organic acids were found. However, malate levels did increase to 27% of the total organic acid fraction for both corn lines when Al-stressed from values of 18% and 22% for A632 and W64, respectively, in non Al-stressed plants. It is of interest to note that the citrate level of A632 diminished substantially upon Al-stress whereas in W64 the concentration of citrate was maintained at approximately the level prior to Al-stress. It is apparent that the distinguishing feature between the Al-tolerant W64 and the Al-sensitive A632 is the ability of W64 to maintain higher levels of organic acid in the presence of toxic Al ions.

In studies on Al-induced changes of organic acid levels in hydroponically cultured sorghum plants both increases (Cambraia et al. 1983) and decreases (Guerrier 1982) of total organic acid levels in root tissue have been reported. This discrepancy may stem from differences in the age at which the seedlings were stressed, the composition of the culture solution, and the manner in which the Al stress was applied. The use of younger (1 week old) corn seedlings in our work in comparison to 2-3 week old sorghum (Cambraia et al. 1983) and 4 week old sorghum

(Guerrier 1982) plants used in previous work may account for the enhanced susceptibility of our plant material to Al stress. Moreover, plant growth in Hoagland solution (Cambraia et al. 1983, Guerrier 1982) may diminish the accessibility of Al to the plant roots as opposed to the simplified medium consisting only of 0.25 mM $CaSO_4$ solution used in our studies. Finally, in our work the corn seedlings were supplied with fresh culture medium daily both prior to and during Al stress (0.15 mM or 4 ppm) which lasted 3 days. It is noteworthy to mention that sorghum roots stressed for 24 hr with 10 ppm Al showed an overall 15% decrease in the organic acid content of their roots (Guerrier 1982); qualitatively this observed decrease is in accord with our own findings. However, increased levels of organic acids were found in sorghum roots when the Al stress was applied over a concentration range of 2-10 ppm through the culture medium over a 5 day period without a daily change of Al-containing nutrient solution. In comparison to the sorghum studies our Al stress regime provided a constant Al stress for the longest time duration and is probably a reasonable simulation of Al availability to plants from the soil solution.

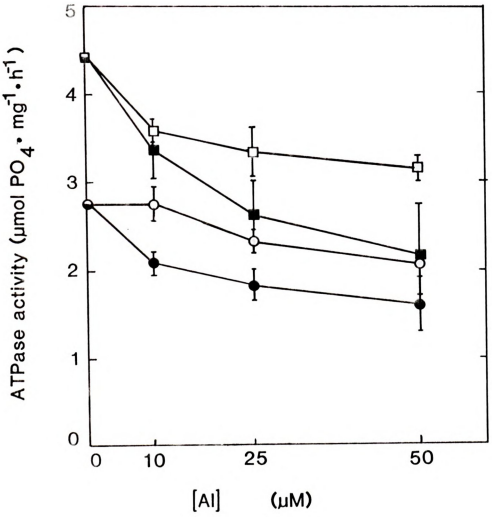
Aluminum inhibits membrane-bound ATPase activity

A significant amount of Mg^{2+}-dependent ATPase activity was found in the plasma membrane enriched microsome fraction of the non Al-stressed A632 hybrid. ATP hydrolyzing activities of 2.75 µmol Pi/mg protein·hr and 0.20 µmol Pi/mg protein·hr were observed at pH 6.5 and 22°C in the presence and absence of 3 mM Mg^{2+}, respectively, demonstrating the Mg^{2+} dependence of the enzyme and the low background of non-specific ATP hydrolyzing activity. Application of 50 mM KCl stimulated the enzymatic

activity to 4.45 µmol Pi/mg protein·hr, representing a 1.6-fold increase in enzyme activity (Note: the Mg^{2+}-ATPase activity is not subtracted out of the K^+ stimulated activity of the enzyme). The Mg^{2+}-dependent and K^+-stimulated ATPase activities are in agreement with previously published plasma membrane-bound ATPase activities from corn roots (Leonard and Hotchkiss 1976). The addition of sodium orthovanadate, a known plasma membrane-bound ATPase inhibitor (Bowman et al. 1978), at a concentration of 100 µM per ml of assay mixture inhibited both the K^+-stimulated and Mg^{2+}-ATPase activities by 61% and 47%, respectively. The addition of $CaCl_2$ at concentrations of 0.5 mM and 1.0 mM inhibited the Mg^{2+}-ATPase activity by 42% and 60%, respectively, and is in agreement with the known sensitivity of Mg^{2+}-ATPase to the inhibitory effects of Ca^{2+} (Leonard and Hotchkiss 1976, DuPont et al. 1982). The Mg^{2+}-ATPase was insensitive to oligomycin, an inhibitor of mitochondrial ATPase (Leonard and Hotchkiss 1976) at concentrations that effectively inhibited mitochondrial ATPase (data not shown). The Mg^{2+}-ATPase containing plasma membrane enriched microsome fraction had an antimycin A-insensitive NADPH cytochrome C reductase (an endoplasmic recticulum marker enzyme) activity of 0.018 µmol cytochrome C reduced/mg protein·min as compared to 0.010 µmol cytochrome C reduced/mg protein·min for plasma membrane preparations from oat roots (Hodges and Leonard 1974). The above data thus indicate that the microsome fraction used for enzymatic assays is of plasma membrane origin and is relatively free of other contaminating subcellular membranes.

The Mg^{2+}-ATPase and the K^+-stimulated Mg^{2+}-ATPase activity were both inhibited by the addition of Al to the assay mixture (Figure 1). The addition of 10, 25 and 50 µM concentrations of Al to 20 µg membrane

Figure 1. Application of citrate reduces the aluminum-related inhibition of ATPase activity in root plasma membranes isolated from the Al-sensitive maize hybrid, A632Ht. The Mg^{2+}-ATPase activity (μmol P_i/mg protein·h) was measured at pH 6.5, 22°C, in the presence of Al ions added (●), or with 100 μM citrate present prior to Al addition (○). The K^+-stimulated Mg^{2+}-ATPase activity was assayed with Al ions added (■), or with 100 μM citrate present prior to Al addition (□).


ATPase activity (µmol PO_4 • mg^{-1} • h^{-1})
5
4
3
2
1
0
0
10
25
50
[Al] (µM)

protein per ml assay mixture reduced Mg^{2+}-ATPase activity by 25%, 34% and 42%, respectively (Figure 1). As with VO_4^{3-} sensitivity the K^+-stimulated Mg^{2+}-ATPase activity was more sensitive to Al than the Mg^{2+}-ATPase activity. In the presence of 50 mM KCl and with the addition of 10, 25 and 50 µM Al the K^+-stimulated activity was inhibited 25%, 41% and 54%, respectively (Figure 1). As a control when 50 µM of Al was added to the complete enzyme reaction mixture that had been spiked with 30 µM phosphate no interference by Al with color development in the phosphate assay was observed.

Another important consideration is the relative molar ratios of Al to Mg^{2+} and ATP used in the enzyme assay since Mg^{2+}-ATP is the active substrate for the ATPase. Equimolar ratios of Mg^{2+} and ATP at a final concentration of 3 mM were used in the ATPase assay. At the Al concentrations (10, 25 and 50 µM) employed, ATP and Mg^{2+} are present in excess of Al by 300-, 120- and 60-fold, respectively. The large excess of Mg^{2+} and ATP over the low concentrations of Al employed in conjunction with the amount of membrane protein used in the enzyme assay (20 µg/ml) leads us to believe that the observed inhibition by Al is the result of Al perturbation of the ATPase and/or the membrane rather than inhibition of the enzyme by Al-ATP (Womack and Colowick 1979).

Organic Acids Reduce Aluminum Inhibition of ATPase Activity

The addition of 100 µM of citrate to the reaction mixture containing 20 µg membrane protein prior to the addition of Al ions reduced the Al inhibition of ATPase activity (Figure 1). Mg^{2+}-ATPase activity in the presence of 100 µM citrate and Al at concentrations of 10, 25 and 50 µM was inhibited by 0%, 15% and 26%, respectively whereas K^+-stimulated

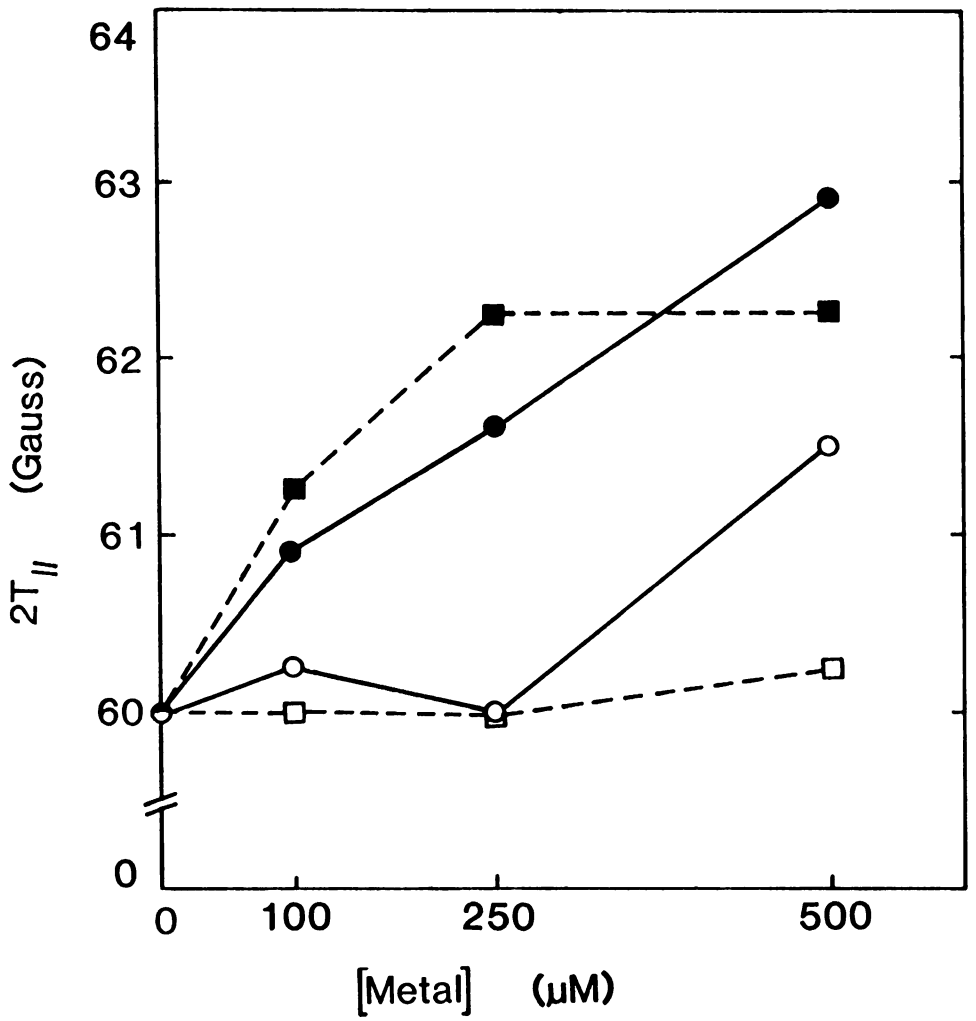
activity was reduced 21%, 25% and 29% under identical conditions. It appears that citric acid, when present in excess of Al ions can reduce the Al inhibition of Mg^{2+}-ATPase and K^{+}-stimulated Mg^{2+}-ATPase activity. These findings are in agreement with earlier studies showing the protective effects of organic acids against Al inhibition of calmodulin stimulated phosphodiesterase activity (Suhayda and Haug 1985).

Citrate Prevents Aluminum-Induced Membrane Changes

The spin label 5-doxyl stearic acid (5-DS) was incorporated into plasma membrane microsomes isolated from the Al-sensitive cultivar, A632. The probe was used to monitor the mobility of lipid acyl chains just below the polar head group region at the membrane surface. The rotational mobility of 5-DS in the membrane is related to the value of the hyperfine splitting parameter, 2T//. A large value of 2T// indicates a reduction in probe mobility in the membrane whereas a smaller value of 2T// is indicative of the spin label in a more fluid microenvironment.

In the absence of added metal ions and measured at pH 6.5 and 22°C, 5-DS was in a fluid microenvironment in the membrane having a 2T// value of 60 Gauss (Figure 2). The titration of up to 500 µM of Al ions onto the membrane (100 µg total membrane protein) increased 2T// values indicating a reduction in 5-DS mobility and presumably the binding of Al ions to lipid headgroups (Figure 2). The binding of metal ions to polar lipid headgroups at the membrane surface has been shown to reduce headgroup mobility as measured by spin labeling techniques (Caldwell and Haug 1981b, Gordon et al. 1978). This reduction in mobility is transmitted to the lipid acyl chains; the effect being attenuated as the nitroxide reporting group is moved deeper into the membrane bilayer

Figure 2. Metal-induced changes of the hyperfine splitting paramenter, $2T_{//}$, of plasma membranes isolated from the Al-sensitive maize hybrid, A632Ht. The membranes were first labelled with a 5-doxyl stearic acid spin probe, then EPR spectra were recorded in samples of 100 µg membrane protein in 100 µl, 10 mM Pipes buffer, pH 6.5, at 22°C. Increasing values of $2T_{//}$ represent enhanced restraints in the mobility of the spin label. Aluminum-induced changes in $2T_{//}$ were measured in the absence of citrate (●), or with 500 µM citrate present prior to aluminum addition (○). Similarly, calcium-related changes of $2T_{//}$ were measured in the absence of citrate (■), or with 500 µM present prior to calcium addition (□).


64
63
62
61
60
0
$2T_{//}$ (Gauss)
0
100
250
500
[Metal] (μM)

(Caldwell and Haug 1981b). Calcium-induced membrane fluidity changes are thought to result from the interaction of calcium ions with the polar lipid headgroups at the membrane surface (McLaughlin et al. 1971, Gordon et al. 1978, Caldwell and Haug 1981b). The addition of up to 250 μM of Ca ions to the root plasma membrane preparations of A632 under conditions identical to the Al titration resulted in the reduction of 5-DS mobility (Figure 2). Unlike the Al titration however the higher concentrations of Ca ions (500 μM) failed to further reduce the mobility of the spin label.

The preaddition of 500 μM of citrate to the plasma membrane preparations was effective in preventing both Al- and Ca-induced membrane rigidification. At an Al concentration of 500 μM representing a [citrate]/[Al] molar ratio of 1, application of Al ions reduced the hyperfine splitting parameter but only by half as much as in the absence of citrate (Figure 2). Ca-induced 5-DS immobilization was virtually completely prevented at all Ca concentrations used by the preaddition of 500 μM citrate (Figure 2).

Organic Acids Endogenous to Corn Root Tissue Mediate the Removal of Aluminum from Calmodulin

Calmodulin, a pivotal protein for calcium regulation (Klee et al. 1980), has been shown to undergo Al-induced conformational changes that result in the loss of its regulatory activity (Siegel et al. 1982, Siegel and Haug 1983a). This Al-induced inactivation of CaM has been postulated to be a key subcellular lesion in the broadly defined syndrome of Al toxicity (Siegel and Haug 1983a). Since we have previously demonstrated that certain organic acids can have a protective role against Al-induced

conformational changes in CaM (Suhayda and Haug 1984), we tested the organic acids found in corn root tissue for their ability to reverse Al-induced conformational changes in CaM (Table 2). Citrate reduced the Al-dependent increase in fluorescence of the ANS-CaM complex by 100%, or to the original value prior to Al addition to the protein, in agreement with previous results (Suhayda and Haug 1985). Citrate was followed in decreasing order of efficiency by malonate > malate > quinate > trans-aconitate, fumarate > succinate. The Al-chelating ability of the individual organic acids with respect to Al removal from CaM appears to correlate with their stability constants with Al. For example, the stability constants for the monomeric Al-citrate chelate and the Al-malate chelate are $5.0 \cdot 10^{8} M^{-1}$ and $7.2 \cdot 10^{5} M^{-1}$ (Neet et al. 1982), respectively. These values are in accord with the enhanced ability of citrate to chelate Al from CaM. It should also be noted that the value of the Al-citrate stability constant is 50 to 200 times greater than the binding constant derived for the binding of the first mole of Al per mole of CaM (the highest affinity site) which has been estimated to be between $2.5 \cdot 10^{6} M^{-1}$ and $1.0 \cdot 10^{7} M^{-1}$ (Siegel and Haug 1983a). On the other hand, the stability constant for the Al-malate chelate is about one third the value of the estimated Al-CaM binding constant. The smaller decrease in fluorescence of the Al-CaM-ANS complex upon application of malate probably reflects the incomplete removal of Al from CaM with the organic acid removing Al only from low affinity sites on the protein. Finally, we have previously shown that the titration of CaM with Ca in the presence of citrate (at a [citrate]/[CaM] molar ratio of 10:1) did not prevent Ca-induced changes in hydropholic surface domains or the observed increase in α-helix content of the protein (Suhayda and Haug

Table 2. Organic acid-mediated removal of aluminum ions from calmodulin.

Organic acid	Percentage of reduction in I_o
Citrate	100
Malonate	64
Malate	36
Quinate	28
t-Aconitate	18
Fumarate	18
Succinate	0

Final calmodulin and ANS concentrations of 10 and 2 µM, respectively, in a total volume of 2 ml, in 10 mM Pipes buffer, pH 6.5. Al ions were added to the ANS-calmodulin complex to a molar ratio of 3:1 for [Al]/[calmodulin], and the resulting fluorescence intensity, I_o, at 490 nm, assigned the 100% value. The respective organic acid was then added at a molar ratio of 9:1 for [acid]/[Al], and the decrease in ANS fluorescence intensity determined 30 min after organic acid application. SD about 5%.

1985). These findings again correlate with differences between the stability constant of the Ca-citrate chelate having a value of $3.2 \cdot 10^{3} M^{-1}$ (Martell and Smith 1977) and a Ca-CaM binding constant of $1.0 \cdot 10^{6} M^{-1}$, derived for the binding of the first mole of Ca per mol of CaM (Siegel and Haug 1983a), revealing a 300-fold higher affinity of Ca for CaM than for citrate.

Discussion

The Al-tolerant corn line, W64, maintains higher levels of organic acids especially malate and transaconitate when stressed with Al ions than the Al-sensitive line, A632 (Table 1). The possible lower affinity of Al for trans-aconitate, as measured by its ability to remove Al from calmodulin (Table 2), may be partially compensated for by the high concentrations of trans-aconitate in root tissue. In corn root tissue trans-aconitate accounts for approximately 55% of the total organic acid content (Table 1). Concentrations of aconitate, malate and citrate in plant tissues have been estimated as high as 10 mM; the local concentrations dependent upon compartmentation, age of cells, and distance from the root tip (MacLennan et al. 1963). The relatively strong affinity of Al for malate as indicated by a stability contant of $7.2 \cdot 10^{5} M^{-1}$ (Neet et al. 1982) in conjunction with the observed high concentrations of this organic acid in root tissue suggests a potential role for malate in sequestering Al intracellularly. As in studies on sorghum (Cambraia et al. 1983), we found 1.5-fold higher malate concentration in the Al-tolerant corn cultivar, W64, when compared with the Al-sensitive cultivar, A632, upon application of Al-stress (Table 1). The higher malate concentrations in W64 may therefore constitute a basis for Al-tolerance in this corn line.

Citrate forms a strong chelate with Al typified by a stability constant of $5 \cdot 10^{8} M^{-1}$ (Neet et al. 1982) which is about 700-fold greater than the corresponding value for the Al-malate complex. Although citrate was present in appreciably lower concentrations in corn root tissue compared with transaconitate and malate, the high stability constant of the Al-citrate complex coupled with its observed stability over the physiological pH range (Karlik et al. 1983) suggests a role for the chelation of Al by this organic acid as a means to prevent the binding of Al ions to key subcellular components (Suhayda and Haug 1985). Additional data collected from young meristematic maize root tissue revealed that most of the intracellular organic acid was associated with metablically active turnover pools while in progressively older, vacuolated tissue larger amounts of organic acid were associated with non-metabolic pools suggesting compartmentation of the acids in the vacuole and/or the cytoplasm (MacLennan et al. 1963). The subcellular concentrations of individual organic acids may be strongly influenced by their association with metabolic or non-metabolic pools within individual compartments of the cell.

Strong circumstantial evidence exists in support of the hypothesis that organic acids through the chelation of Al provide a mechanism for preventing Al toxicity in plants. For example, the addition of Al-citrate or Al-EDTA chelates to solution cultured corn plants did not induce symptoms of Al toxicity or alter the distribution of essential ions in plant tissues in comparison to plants treated with free Al salts (Bartlett and Riego 1972). More recently, suspension cultured carrot cells tolerant to Al were found to release more citrate into the culture medium than Al-sensitive cell lines (Ojima et al. 1984). Al-citrate

complexes have also been identified in the heartwood of an Adinandra brassii tree growing on bauxite containing soils (Price and Worth 1975).

Our membrane studies indicate that Al and Ca ions rigidify membrane lipids probably by binding to negatively charged phospholipid headgroups. The difference in the affinity of Ca for citrate, characterized by a stability constant of $3.2 \cdot 10^{3} M^{-1}$ (Martell and Smith 1977), compared with that of Ca for anionic phospholipids apparently explains the ability of citrate to completely prevent Ca-induced membrane fluidity changes as monitored by spin probes. Since the relative strength of cation binding to phospholipids seems to be proportional to the ion's electric charge (Haug 1984), Al ions should bind to the membrane more tightly than Ca ions. This is also illustrated by our observation that the Al-induced decrease in membrane fluidity is diminished at a [citrate]/[Al] molar ratio of 1 (Figure 2). Furthermore, at acidic pH values application of Al ions to prokaryotic plasma membranes produced profound changes in membrane fluidity and in temperatures indicative of membrane lipid phase changes, at Al concentrations as low as 10 µM (Vierstra and Haug 1978). Pronounced ultrastructural changes were found in thylakoid membranes upon exposure of cyanobacteria to micromolar Al concentrations at slightly acid pH values (Pettersson et al. 1985). Recently, application of aluminum salts have been implicated in producing a subtle lipid rearrangement which, in turn, facilitates the peroxidative action of iron (II) salts applied to brain phospholipid liposomes at acid pH values (Gutteridge et al. 1985). Taken together with our results, these findings suggest that Al-stressed corn roots may have a lower organic acid content than non-stressed roots as a result of Al-induced injury to

root plasma membranes which facilitates leakage of essential metabolites out of the root.

The initial Al-induced decrease in membrane fluidity (Figure 2) coincided with the Al-related inhibition of the Mg^{2+}-ATPase and the K^+ stimulated enzyme activity (Figure 1). Several mechanisms for the inhibition of ATPase activity by Al ions are possible. First, Al ions bind to membrane lipids, in particular the "boundary lipids" thereby disrupting the fluid membrane microenvironment required by the enzyme. These types of metal interactions have been suggested in the observed Ca inhibition of the Na^+, K^+-ATPase in rat liver microsomes (Gordon et al. 1978). The interrelationship between enzymatic protein and lipids is also illustrated by observations that delipidation of membrane-bound Mg^{2+}-activated ATPase in cucumber roots clearly inhibited the enzymatic activity (Matsumoto and Kawasaki 1981). Second, Al ions may act directly on the enzymatic protein or on proteins involved in the stimulation of the enzyme as with the calmodulin-dependent Ca^{2+}, Mg^{2+}-ATPase activity of barley root plasma membrane vesicles (Siegel and Haug, 1983b). In this case Al-induced alterations of calmodulin's structure were correlated with diminished formation of the transmembrane potential in the presence of ATP. Electrophysiological measurements also show that upon application of Al ions to root cells, the transmembrane potential is lowered (Etherton et al. 1983). Third, since we employed 10-50 µM Al concentrations, metal-related alterations of the membrane surface charge were probably not involved in influencing the membrane-bound enzymatic activity (Wojtezak and Nalecz 1979).

The presence of citrate in slight excess of Al in the ATPase reaction mixture reduced the Al inhibition of both the Mg^{2+}-ATPase and K^+

stimulated enzymatic activity, presumably through Al-chelation by the organic acid. Conceivably in the presence of higher citrate concentrations the Al interference with ATPase activity could be completely alleviated.

In conclusion, our data support the hypothesis that the presence of organic acids especially malate and citrate at sufficient concentrations in plant roots can serve to prevent or reduce the toxic effects of Al at the cellular level.

Acknowledgements

This work was supported by a grant, PCM-8314662, from the National Science Foundation, and by a gift from Pioneer HiBred International.

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CHAPTER VII

Summary

The data presented in this dissertation indicate a strong interaction between Al ions and calmodulin (CaM) that lead to alterations in the protein's conformation and result in the impairment of CaM's regulatory activity. Once Al ions are bound to CaM only a partial restoration of the protein's conformation to its original metal free state is possible by the addition of aluminum (Al) chelating agents. This irreversibility suggests that Al ions either form stable complexes with CaM that can exist in the presence of chelators or that Al denaturation of the protein results in the rearrangement of water molecules and/or hydrogen bonding sites within the protein that prevent its return to the metal free conformation. Complete protection of CaM from Al is evident when strong Al chelators such as citrate are present with CaM prior to the addition of Al ions. Carboxyl and hydroxyl rich ligands like organic acids and catechol were especially effective in chelating Al ions from CaM, as were ATP and F^- ions. The protective and restorative effects of citrate against Al-induced conformational changes in CaM were reflected in the partial restoration of CaM stimulation of cGMP-dependent phosphodiesterase. Taken together these results imply an important role for organic acids in the protection of key subcellular components against Al inactivation.

The root plasma membrane represents the primary barrier through which Al ions must pass to enter the cell cytoplasm. The addition of 10-50 μM Al ions to a plasma membrane-enriched microsome fraction from corn roots inhibited the Mg^{2+}-ATPase activity by about 40% at the highest Al concentration used. The inhibition of ATPase activity was paralleled by a substantial decrease in plasma membrane fluidity as

measured by lipophilic spin labels. Once again the preaddition of citrate in excess of Al to plasma membrane fractions prevented Al inhibition of Mg^{2+}-ATPase activity as well as Al-induced membrane fluidity changes. At nearly equimolar citrate:Al concentrations the Al effects on ATPase activity and membrane fluidity were strongly attenuated by the presence of this organic acid. It is apparent that high cytoplasmic concentrations of organic acids could afford protection against Al-induced changes in cellular and subcellular membranes.

A continuation of research in the area of Al interactions with subcellular components is important to identify other possible cellular targets of Al ions. In this respect it would seem worthwhile to use anion exchange chromatography as a tool to fractionate acidic cytosolic proteins using an Al solution gradient. This technique could reveal other cellular proteins whose conformation and/or physical properties are sensitive to Al ions. Another study employing Al-tolerant and Al-susceptible corn seedlings cultured in Al containing solutions should examine Al-induced changes in root plasma membrane properties such as membrane-bound ATPase and membrane fluidity. This may provide an indication of the types of membrane properties that could have some adaptive value in response to Al stress.

APPENDIX I

Heavy Metal Inhibition Of The Barley Root Plasma Membrane-Bound Ca^{2+}-ATPase And Its Reversal By Monovalent Cations

The Science of the Total Environment, 28 (1983) 249–256
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

HEAVY METAL INHIBITION OF BARLEY ROOT PLASMA MEMBRANE-BOUND Ca^{2+}-ATPase AND ITS REVERSAL BY MONOVALENT CATIONS

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ABSTRACT

When applied in the physiological pH range at 16°C, divalent cations such as Cd^{2+}, UO_2^{2+}, and Ca^{2+} inhibit the Ca^{2+}-ATPase activity associated with plasma membrane microsomes isolated from roots of barley (Hordeum vulgare L. cv. Conquest). This inhibition results from a restriction of the motional freedom of the polar head groups which partially form the enzyme's microenvironment. Moreover, this inhibition could be removed by monovalent cations, such as Na^+, which displace the divalent cation from its binding site at the lipid head group. These results suggest that acidic phospholipids constitute part of the ATPase microenvironment.

INTRODUCTION

The root plasma membrane interfaces the root cell with its biosphere and serves both an architectural and a functional purpose. These membranes perform such functions as the regulation of the exchange of metabolites between the cell and the environment, and the maintenance of electrochemical gradients. Typically the gross organizational features of a membrane are described by the fluid mosaic model [1]. The lipid amphiphiles are arranged as a bilayer leaflet where the hydrophilic moiety stays in contact with the aqueous phase. The apolar lipid moiety is shielded from the polar environment and may interact with membrane proteins. Recently considerable attention has been directed to the careful study of lipid-protein interactions which can influence enzymatic activities of biological membranes [2].

Abbreviations used: EPR = electron paramagnetic resonance; MES = morpholinoethanesulfonic acid; $2T_{//}$ = hyperfine splitting parameter; 5 NS = 5-nitroxy stearic acid, [2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl]; CAT_{12} = N, N-dimethyl-N-dodecyl-N-tempoylammonium bromide; Tris = tris-(hydroxymethyl)aminomethane; ETC = equivalent temperature change.

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Since the plasma membrane of root cells operates as an interface device, certain components of a given environment, e.g. cadmium, may induce structural and functional perturbations which may be injurious to the plant. For instance, the concentration of cadmium in non-polluted soil ranges from 0.01 to 7 mg kg^{-1} [3]. This metal is readily taken up by plants. The cadmium content of corn roots was about 1 mg kg^{-1} for the control and 32 mg kg^{-1} for plants exposed to 5 mg kg^{-1} cadmium [4]. The uptake and redistribution of cadmium by excised barley roots involve various processes [5]: (a) the largest amount of cadmium is reversibly bound to the root tissue and can be displaced by divalent cations, (b) irreversible binding, and (c) diffusion of cadmium across the cell membrane.

Because next to nothing is known about plant membranes as permeability barriers for heavy metals, experiments were designed to investigate the effects of heavy metals, especially those of cadmium, on physico-chemical properties of barley root plasma membranes. Barley was selected because:

(a) it is representative of a crop plant rather universally distributed,
(b) it is fairly tolerant towards cadmium which allows future experimentation on molecular determinants responsible for cadmium tolerance, and
(c) its root plasma membrane has been isolated and the kinetics of the associated membrane-bound ATPase have been characterized [6].

In this report results are presented which demonstrate that cadmium can modify the activity of the membrane-bound Ca^{2+}-ATPase by binding to lipids. Moreover, under certain experimental conditions, monovalent cations can reverse the cadmium-induced inhibition of the membrane-bound enzyme.

EXPERIMENTAL

Plant material and isolation

Barley seeds (Hordeum vulgare L. cv. Conquest) were germinated and grown over an aerated solution of 0.25 mM $CaSO_4$ (pH 6.5) in the dark at 16°C. A plasma membrane-rich fraction was isolated from 5 day old seedlings as described previously [6]. Freeze fracture electron microscopy had indicated a rather homogeneous material composed of vesicles. This material was used immediately for ATPase assays and electron paramagnetic resonance (EPR) experiments.

ATPase assay procedures.

The enzymatic activity of the Ca^{2+}-ATPase was determined in a 1 ml reaction volume, containing 20 mM Tris-MES buffer (pH 6.5), 1 ml Tris-ATP, and 1 mM $CaCl_2$, and, depending on the experiment to be performed, various concentrations of other cations, generally added as their chloride salts. For the experiments protein concentrations of 2 µg ml^{-1} were employed. The assay temperature was 16°C, which is the optimal growth temperature for barley plants.

Lipid analysis.

Lipids were isolated from barley root plasma membranes following a procedure described recently [7]. Subsequently the lipids were analyzed by thin layer chromatography on Sil-G-25 plates (Brinkman Instruments, Inc., Wesbury, NY, USA) using solvent system A [8]. For visualization of the lipids, the dried chromatograms were sprayed either with a solution of 3% cupric acetate in 8% phosphoric acid [8], or with a modified molybdenum blue reagent by Zinzadze [9].

EPR experiments.

EPR spectra were obtained with a Varian X-band spectrometer, model E-112. The sample temperature was 16±0.3°C and was monitored with a copper/constantan thermocouple. All spectra were recorded at power and modulation amplitude settings which had been found to be below those causing saturation or line-width broadening. The spin labels, 5 NS and CAT_{12}, were added to the membrane suspensions at a concentration of about 10 nmol/mg membrane protein. The hyperfine splitting parameter, $2T_{//}$, is indicative of motional properties of the spin probe located in the membrane. A high value of $2T_{//}$ is associated with a restricted motion or increased rigidity [10]. To express conveniently cation-induced membrane changes, the ETC value was introduced, which describes the change in $2T_{//}$ caused by the addition of the cations. This value expresses the temperature change necessary to match the measured change in membrane lipid head group mobility resulting from cation application. The surface potential, Ψ_s, was calculated from the first derivative EPR spectra obtained from membranes labelled with the charge sensitive spin probe, CAT_{12} [11]. Spin probes were added to the lipid dispersions so that the spin label concentration was less than 0.1% of the sample's lipid weight. Insertion of the spin probes was facilitated by a 15 min sonication in an ultrasonic cleaner.

Chemicals.

Vanadium-free ATP, Tris, and MES were purchased from Sigma Chemical Co (St. Louis, MO, USA). The sodium salt of ATP was converted to its Tris salt prior to use. Synthetic phosphatidyl choline dipalmitoyl, phosphatidyl serine (bovine brain), phosphatidyl inositol (soybean) were also purchased from Sigma Chemical Co , and were of the highest grade available. The charge sensitive spin probe, CAT_{12}, was synthesized in our laboratory according to published procedures [11]. All other chemicals were bought from various commerical sources and were also of the highest degree of purity available.

RESULTS

Representative EPR spectra of spin labelled membrane vesicles, recorded at 16°C, are presented in Fig. 1. Such an EPR spectrum may be interpreted in terms of two superimposed spectral components, one corresponding to a relatively mobile spin population. Previous experiments had demonstrated that the immobilized signal appeared to arise from spin probes associated with membrane lipids [12]. Furthermore, the inhibition of the Ca^{2+}-ATPase at higher levels of Ca^{2+} seemed to be

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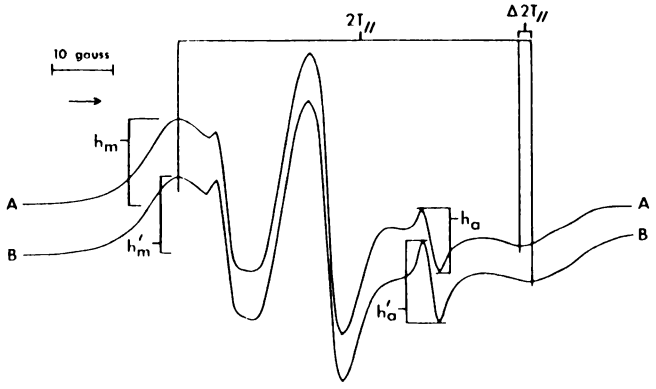

Figure 1. Typical EPR spectra of barley root plasma membranes spin labelled with CAT_{12}. The partition coefficient, P, for the probe partition between membrane and the aqueous environment was determined from respective peak heights [ref. 11]. Spectrum B was recorded after the addition of cations. A change in the surface potential was calculated from the P values [ref. 11].

correlated with the calcium-induced restriction in the polar head group mobility of lipids surrounding the enzymatic protein [12].

The effects of cation addition on the root plasma membrane, labelled with spin probes, were evaluated by determining the hyperfine splitting parameter, $2T_{//}$, and the ETC value (Fig. 2). The $2T_{//}$ value may be considered an indicator of "lipid fluidity" to express the relative motional freedom of lipid molecules where interpretations are based on relative changes in the EPR spectra [10].

Analysis of the cation interactions with the root plasma membrane showed that Sr^{2+} addition had virtually no influence on the rigidity of the membrane expressed by a constant ETC value - and on the enzymatic activity (Fig. 2A). The surface potential decreased, which was consistent with the observation that Sr^{2+} mainly screens surface charges [13]. UO_2^{2+} inhibited the enzymatic activity concomitant with a reduction in lipid polar head group mobility. At higher concentrations this divalent cation screened the surface charges (Fig. 2B). Hg^{2+} strongly inhibited the Ca^{2+}-ATPase probably by binding to groups located at the enzymatic protein such as thiol groups. Only at higher concentrations did Hg^{2+} affect the surface potential and the ETC value noticeably (Fig. 2C). In contrast to Hg^{2+} ions, application of increasing levels of Cd^{2+} ions inhibited the enzymatic activity concomitant with a restriction in lipid polar head group mobility; the membrane surface charge was also changed (Fig. 2D).

In the presence of a basal concentration of 1 mM Cd^{2+}, increasing levels of NaCl and ethanolamine could partially reverse the cadmium-induced inhibition of the ATPase activity. This reversal proceeded parallel to a gradually enhanced motional freedom of the polar head groups (Fig. 2 E,F). This restoration of the enzymatic

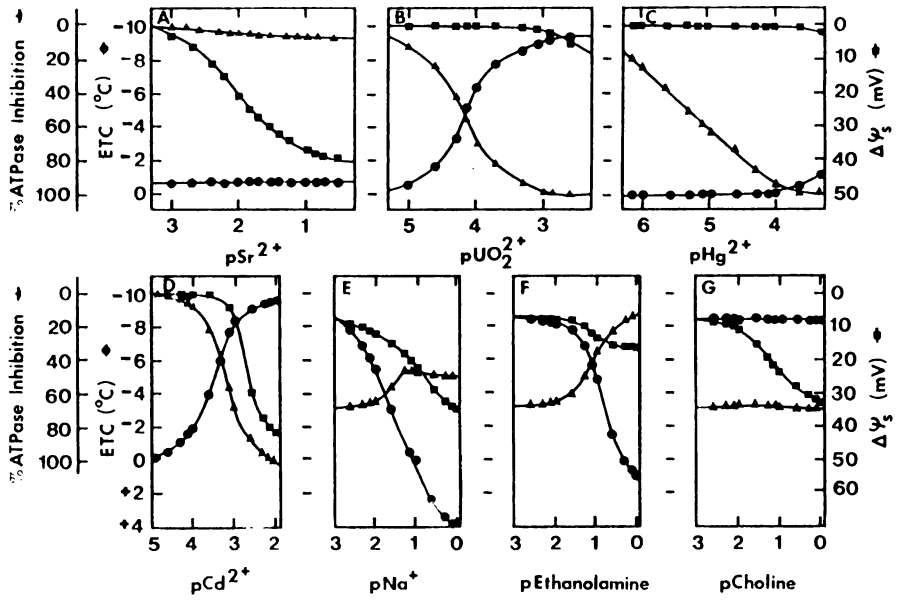

Fig. 2. The influence of cations on the equivalent temperature change, ETC, the membrane surface potential, ψ_s, and the Ca^{2+}-ATPase activity associated with plasma membranes isolated from barley roots. All measurements were carried out at pH 6.5 and at 16°C, the optimal growth temperature for barley plants. The Ca^{2+}ATPase activity represents the enzymatic activity (100%) determined in the presence of 1 mM $CaCl_2$. The respective monovalent concentrations (E,F.G) were added to the membrane vesicles in the presence of 1 mM Cd^{2+}. The abscissae list molar concentrations. The ATPase activities had standard errors of less than 5% of the given values.

activity was more effective in the case of ethanolamine. In contrast to the bulkier ethanolamine, Na^+ ions apparently have the ability to virtually neutralize the electrostatic environment of the ATPase, to a certain extent necessary for proper enzyme performance. The membrane surface potential was lowered by the application of Na^+, ethanolamine and choline (Fig. 2 E,F.G). On the other hand, choline was essentially ineffective to reverse the cadmium-induced inhibition of the enzyme and to remove the restraint in motional freedom. The cadmium-induced inhibition of the enzymatic protein was independent of the membrane concentration thus suggesting the existence of a reversible inhibition.

The Ca^{2+}-ATPase activity was further examined to explore the relationship of activity to pH and cadmium concentration (Fig. 3). At neutral and slightly acidic pH values, the enzymatic activity was increasingly inhibited with higher levels of Cd^{2+}. At slightly alkaline pH values, certain lower concentrations of Cd^{2+} appeared to stimulate the ATPase activity (Fig. 3).

Vesicles prepared from dipalmitoylphosphatidyl choline, -serine, and -inositol, were spin labelled with 5 NS, and examined by EPR spectroscopy at 16°C and pH

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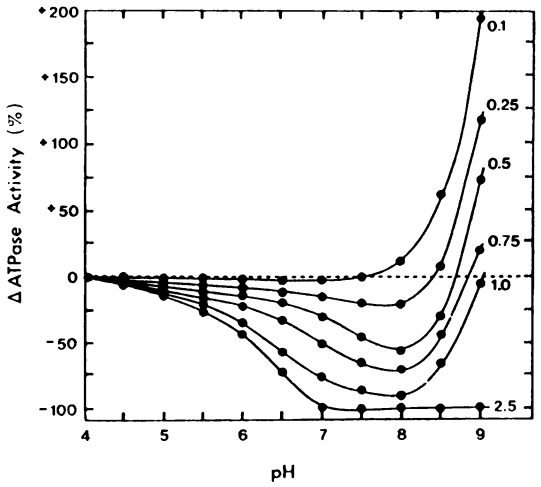

Fig. 3. The effect of pH and millimolar Cd^{2+} concentrations on the Ca^{2+}ATPase activity, measured at 16°C, in the presence of 1 mM $CaCl_2$.

6.5, over a range of cadmium concentrations. Preliminary results indicated a rigidification of the lipid vesicles with increasing cadmium concentration. Phosphatidyl serine and -inositol vesicles were strongly rigidified in the presence of cadmium concentrations greater than 0.1 mM. Phosphatidyl choline vesicles exhibited the greatest increase in rigidity at cadmium concentrations between 0 and 1 μM; however, at cadmium concentrations in the millimolar range the rigidity of phosphatidyl choline vesicles varied only slightly as compared to that of the other lipids studied.

In preliminary studies, thin layer chromatography of the lipids isolated from barley root plasma membranes showed that phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidic acid were the principal membrane phospholipids.

DISCUSSION

Based on the data presented in this article, several general statements can be made about the interaction of metal cations with the plasma membrane isolated from barley roots. First, when applied in the physiological pH range, cations such as Cd^{2+} and UO_2^{2+} inhibit the Ca^{2+}-ATPase presumably by restricting the mobility of the polar head groups of lipids surrounding the enzymatic protein. These findings are in accord with those demonstrating that higher levels of Ca^{2+} inhibit the ATPase and restrict lipid mobility, although $CaATP^{2-}$ is the active substrate for this enzyme [6]. Second, the observations that Ca^{2+} [12], Cd^{2+} and UO_2^{2+} induce isothermal membrane changes which reduce the enzyme's maximal velocity are consistent with findings obtained upon lowering the sample temperature [12]. This is also supported by the experiments with Sr^{2+} indicating that the ATPase activity was not impaired as long as the ETC value remained constant. Third, as evidenced by results

obtained on application of Sr^{2+} and choline to plasma membranes, alterations of the membrane surface charge are presumably not an important factor in the inhibition of the membrane-bound Ca^{2+}-ATPase. Consequently, under our experimental conditions, the local concentration of $CaATP^{2-}$ at the active site is probably not enhanced by a reduction of the membrane surface potential - a mechanism which had been invoked for certain membrane-bound enzymes [14]. Fourth, application of increasing concentrations of monovalent cations, such as Na^{+}, displaced the divalent Cd^{2+} from the lipid polar head group, presumably the phosphodiester group [15]. Following the removal of the divalent cations, the motional freedom of the lipid polar head group is restored which results in an enhanced ATPase activity.

Previous experiments on the temperature dependence of the ATPase activity had indicated that "boundary lipids" (to use an operational expression) play a significant role in the regulation of the barley root ATPase over a range from about 13° to 34°C [12]. Within this temperature region, the Michaelis-Menten constants were virtually temperature-independent. Similar studies have been reported on the involvement of boundary lipids in the regulation of microbial enzymatic proteins [16]. Collectively, the previous information obtained from experiments following Ca^{2+} application [6], and the present results using other cations support the concept of a microenvironment important for the functioning of the Ca^{2+}-ATPase in barley root plasma membranes. Consequently the question can be raised as to the nature of this lipid environment. Presumably a membrane-bound enzyme determines its proper microenvironment by virtue of strong and specific lipid-protein interactions. In the absence of conclusive reconstitution experiments, only speculations are possible regarding some of the constituents of this microenvironment.

At physiological pH values, phosphatidylcholine and phosphatidylethanolamine are isoelectric and carry no net charge. Metal cations have been shown to bind primarily to the phosphodiester group. The question of the stoichiometry of the metal/lipid molar ratio is still a matter of debate [15]. The interaction of cations with acidic phospholipids, e.g., phosphatidic acid and phosphatidylserine which carry a negative charge in the physiological pH range, is stronger than that with phosphatidylcholine [15]. In phosphatidylserine the binding site could be the phosphodiester group, the carboxyl group, or both; in phosphatidic acid the binding site seems to be the phosphate group [15]. Ca^{2+} and Cd^{2+} complexes with dipalmitoyl phosphatidic acid are isomorphous as demonstrated by X-ray diffraction; the phosphate group is involved in the complex formation [17].

In acidic lipids the degree of dissociation depends on the pH and on the ionic strength. Therefore, variations in charge and packing characteristics of the polar group are expected to influence lipid-protein interactions if these types of acidic phospholipids form part of the enzyme's microenvironment. Since the motional freedom of the polar head groups is weakly coupled to the conformational mobility of the lipid acyl chains [18], lipid-protein interactions also occur in the hydrophobic core region.

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The results presented in this article seem to demonstrate that acidic phospholipids constitute part of the enzyme's microenvironment. This hypothesis is based on the following observations: (a) Cd^{2+} inhibition of the enzymatic activity is reversed by monovalent cations consistent with the cation exchange properties of acidic phospholipids; (b) the Cd^{2+}-induced inhibition of the Ca^{2+}-ATPase is modulated by variations of the pH; (c) the apparent deinhibition of the cadmium-induced inhibition of the enzymatic activity at slightly alkaline pH values may be attributed to the ionization state of phosphatidic acid. Above pH $\gtrsim$ 7.3, the second proton of the phosphate group commences to dissociate and therefore the lipid molecule carries two charges. However, the deinhibition is possibly related to the type of cadmium complexes formed at a given pH value. At pH < 8, cadmium exists predominantly as Cd^{2+} whereas $CdOH^{+}$ begins to form around pH 7.5 and is very pronounced at approximately pH 10 [4]; (d) phosphatidic acid is one of the principal phospholipids found in barley root plasma membranes.

Because roots are the point of contact of the plant and soil-borne components of the environment, the plasma membrane is probably the initial target for metal toxicity. An understanding of the entry mode of heavy metals into plants - and later on into the food chain - is fundamental for the evaluation of environmental risk factors. Futhermore, identification of molecular determinants conferring metal tolerance is necessary for the genetic development of future crop plants tolerant for biomass production on polluted soil.

ACKNOWLEDGEMENTS

This work was supported by the US Department of Energy Contract No. DE-AC02-76ER01338.

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