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DISRUPTION OF THE REGULATORY CONFORMATION OF CALMODULIN BY ALUMINUM BINDING: A MOLECULAR BASIS FOR ALUMINUM TOXICITY

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DISRUPTION OF THE REGULATORY CONFORMATION OF CALMODULIN BY ALUMINUM BINDING: A MOLECULAR BASIS FOR ALUMINUM TOXICITY

By

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ABSTRACT

DISRUPTION OF THE REGULATORY CONFORMATION OF CALMODULIN BY ALUMINUM BINDING: A MOLECULAR BASIS FOR ALUMINUM TOXICITY

By

Neal Siegel

The interaction of aluminum ions with bovine brain calmodulin has been examined by fluorescence spectroscopy, circular dichroic spectrophotometry, equilibrium dialysis and by the activation of 3',5'-cyclic nucleotide phosphodiesterase. These experiments show that aluminum binds stoichiometrically and cooperatively to calmodulin. Aluminum binding at a molar ratio of 2:1 to calmodulin suffices to induce major structural changes. Estimates from spectroscopic data indicate that the binding affinity for the first mol of aluminum bound to the protein is one order of magnitude stronger than that of calcium to its comparable site. These estimates agree with a dissociation constant of 0.4 μM derived from equilibrium dialysis experiments. Interaction of aluminum with calmodulin induces a helix-coil transition and enhances the hydrophobic surface area more than does calcium. A molar ratio of 4:1 for [aluminum]/-[calmodulin] completely blocks the activity of the calcium-calmodulin-

dependent phosphodiesterase. Highly hydrated aluminum ions apparently promote solvent-rich, disordered polypeptide regions in calmodulin which, in turn, profoundly influence the protein's flexibility. EPR spectra of spin-labelled calmodulin provide data indicating that aluminum binding causes decreased probe immobilization as compared to the effects of calcium binding. This result of aluminum binding indicates that aluminum-calmodulin is a more random, open polypeptide relative to the structure of calcium-calmodulin. Calorimetric measurements of aluminum binding provide data showing that the first mol of aluminum bound is accompanied by the largest enthalpic change of the three mol bound; the second and third mol of aluminum bound are each entropically driven.

Micromolar concentrations of aluminum ions interfere with calmodulin-stimulated, membrane-bound ATPase activity which plays a role in the maintenance of the transmembrane potential of plasma membrane enriched vesicles isolated from barley roots. At a molar ratio of 3:1 [aluminum]/[calmodulin], the calmodulin-stiumlated enzymatic activity, probably associated with a Ca^{+}+ Mg^{2+}-ATPase is 95% inhibited. Aluminum-induced changes in calmodulin structure are reflected in reduced formation of the membrane potential when assayed with a fluorescent potential probe, oxonol VI. These data strongly suggest that the aluminum-calmodulin complex represents a primary lesion in toxic responses of plants to this metal.

This dissertation is dedicated to my wife, Debbie
for her unfailing love, encouragement and support
and to our puppy, Corduroy
for his loyalty and unselfish devotion.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1

General Introduction

Aluminum is the most versatile metal in use by industrial mankind today. It is the most abundant metal in our world, comprising eight percent of the Earth's crust. Aluminum is also one of the most toxic of the elements; toxic levels range between one and four parts per million for aluminum in solution at low pH. Forty percent of the world's arable soils are unsuitable for plant growth due to acid levels that maintain toxic amounts of aluminum in solution. The potent toxicity of aluminum is clear whether the measure is taken from crop failure or from lakes which can no longer support plant or animal life due to acidification as a result of acid precipitation caused by the burning of fossil fuels by the industrialized world. As indicated, the toxic effects of aluminum are widespread with the symptoms of poisoning and death appearing below five parts per million of the metal in eukaryotic organisms.

In order to understand why aluminum is toxic it is necessary to know something about its physical chemistry. The most unique property of aluminum is that it is bound with water more strongly than any other element of a similar ionic radius. The hydration enthalpy of these bonds is 1144 Kcal/mole (Matheja and Degens, 1971). Six water molecules arranged in a regular octahedron comprise the inner hydration shell; because of its tight association with water, aluminum cannot be considered as a free ion in aqueous solution. In fact, two physical constraints dictate the basic form that the hydrated aluminum ions will have in

solution: pH and concentration (Baes and Mesmer, 1976). At low concentration and pH (less than 4 ppm and pH 3), only monomeric, hydrated aluminum ions of +3 charge exist. If this concentration remains below 60-100 μM (approx. 2-3 ppm) but the pH increases above 3, protons from the inner hydration shell of water molecules will dissociate, and the net charge of the hydrated complex will decrease to neutrality and even become negative. In addition to charge alteration, polymorphic aluminum hydroxides will form and become insoluble. Likewise, if the pH remains low, increased aluminum concentration will promote the formation of polymorphic, insoluble aluminum species. At physiological pH ranging between 6 and 7.5, therefore, only aluminum concentrations between 0 and 4 ppm (0-100 μM) will support monomeric, hydrated aluminum species with net charges ranging between +3 to 0. Thus, the biological toxicity of aluminum is clearly a result of constraints imposed by its solution chemistry.

Although the effects of aluminum toxicity in plants are manifold, ranging from impairment of root elongation (Foy et al, 1978) to chloroplast membrane degeneration (Hampp and Schnabl, 1975) and plasma membrane degeneration in roots (Hecht-Buccholz and Foy, 1981), the most obvious effect on crop plants is impaired calcium uptake, distribution and use (Foy et al, 1978). In recent years calcium involvement as a second messenger by the eukaryotic cells has been shown to be controlled by a ubiquitous, calcium-dependent regulating

protein, calmodulin. Processes ranging from plasma-membrane ATPases (Dieter and Marme, 1981, Caldwell and Haug, 1981) and NAD kinase (Muto and Miyachi, 1977) in plants to calcium dependent phosphodiesterase, ATPases and adenylate cyclase in animals (recent reviews: Cormier et al, 1980, Klee, 1980, Klee et al, 1980, Lin, 1982, Wang and Waisman, 1979) are controlled by calmodulin. Aluminum, known to be a potent calcium antagonist, might exert this antagonism through specific interaction with calmodulin.

Because aluminum is antagonistic toward calcium, a detailed investigation into the physical effects of aluminum on calmodulin and attenuation of the regulation of enzymatic processes under its control was initiated. This allowed a comparison to be made between physical alterations in the structure of calmodulin due to aluminum binding and the assesment of these changes on the regulatory functioning of the protein. In this way, calmodulin could be shown to be a key lesion occuring in the broadly defined syndrome of aluminum toxicity.

Calmodulin isolated from bovine brain was chosen for study because it represents the best characterized calmodulin molecule from all known sources. Its amino acid sequence, order of calcium fill and structural characteristics are well documented. Because of this documentation, even subtle structural changes would be evident as compared with changes occuring in calmodulin from a less studied source.

In order to assess the biological impact of aluminum-induced structural changes in calmodulin, two systems known to be regulated by the protein were assayed for aluminum inhibition; calcium-dependent phosphodiesterase and the Ca^{2+}+ Mg^{2+}-ATPase from barley (Hordeum vulgare var. Conquest) root plasma membranes. The physical techniques used to assess relative structural changes of the protein through interaction with aluminum were independent of the biological activity; these techniques included circular dichroism spectrophotometry, ANS fluorescence spectroscopy, intrinsic tyrosine fluorescence spectroscopy and EPR spectroscopy of spin-labelled calmodulin.

Chapter 2

Aluminum Changes the Conformation of Calmodulin

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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

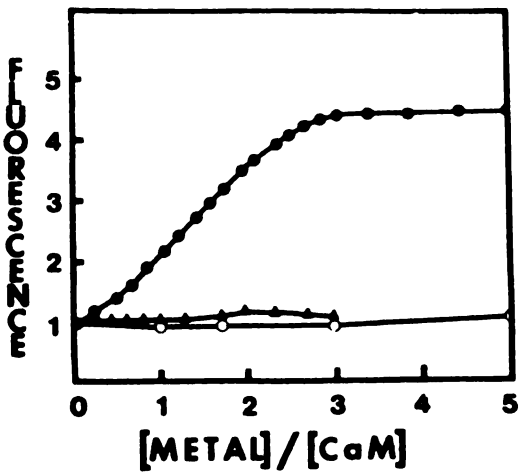

FIGURE 1. ANS fluorescence of bovine brain calmodulin as a function of [metal]/[protein] (mol:mol) ratio. A 7 μM concentration of calmodulin was prepared in 10 mM morpholino propane sulphonic acid (MOPS) buffer, pH 6.5. The concentration of 8-anilino-1-naphthalene sulphonate, ANS, was 2 μM. Excitation 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 1). 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 1. 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.

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Chapter 3

Aluminum Interaction with Calmodulin: Evidence for Altered Structure and Function from Optical and Enzymatic Studies

36

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ALUMINUM INTERACTION WITH CALMODULIN

EVIDENCE FOR ALTERED STRUCTURE AND FUNCTION FROM OPTICAL AND ENZYMATIC STUDIES

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Key words: Calmodulin; Conformational change; Al^{3+}; Metal binding; (Bovine brain)

The interaction of aluminum ions with bovine brain calmodulin has been examined by fluorescence spectroscopy, circular dichroic spectrophotometry and equilibrium dialysis, and by the calmodulin-dependent activation of 3',5'-cyclic nucleotide phosphodiesterase. These experiments show that aluminum binds stoichiometrically and cooperatively to calmodulin. Binding of aluminum at a molar ratio of 2:1 to calmodulin suffices to induce a major structural change. Estimates from spectroscopic data indicate that the binding affinity for the first mol of aluminum bound to the protein is about one order of magnitude stronger than that of calcium to its comparable site. These estimates agree with a dissociation constant of 0.4 μM derived from equilibrium dialysis experiments. Interaction of aluminum with calmodulin induces a helix-coil transition and enhances the hydrophobic surface area much more than calcium does. A molar ratio of 4:1 for [aluminum]/[calmodulin] is sufficient to block completely the activity of the calcium-calmodulin-dependent phosphodiesterase. Highly hydrated aluminum ions apparently promote solvent-rich, disordered polypeptide regions in calmodulin which, in turn, profoundly influence the protein's flexibility.

Introduction

Calmodulin is a ubiquitous, evolutionary highly conserved, multifunctional, calcium-regulating protein which was originally discovered as an activator of cyclic nucleotide phosphodiesterase [1]. Some of the processes in which calmodulin is known to participate involve stimulation of various ATPases, adenylate cyclase, plant NAD kinase [2–4], and the list of calmodulin's roles is being constantly expanded (recent reviews, see Refs. 5–8). Although the detailed mechanisms of calmodulin action remain largely unknown, this molecule is thought to modulate cellular processes in which calcium is the second messenger [6,9]. Four specific calcium-binding sites exist on calmodulin and a detailed investigation of the positive cooperativity exhibited by calcium binding to calmodulin has recently been published [10]. The ability of other metals to compete with calcium for binding to calmodulin has been demonstrated for magnesium, manganese and the sequential binding of terbium [11–13]. Conformational changes induced by calcium binding enhanced the hydrophobic surface exposure of calmodulin [14].

Calmodulin is found in eukaryotic animal and

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ANS, 8-anilino-1-naphthalene sulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; cGMP, guanosine 3',5'-cyclic phosphoric acid.

plant tissues, and in high concentrations in brain tissue, especially in the frontal cortex [4,15]. Antipsychotic drugs and certain neuropeptides have been found to modify calmodulin-mediated neuronal functions [16]. Moreover, changes in calcium levels have been correlated with aluminum accumulation in Alzheimer's disease, which is a neurofibrillary degenerative process in the human central nervous system [17]. In plants, micromolar concentrations of aluminum in the soil and subsequent uptake diminished the rate of root elongation and induced symptoms typical of calcium deficiency [18].

In this report we present results of a study on the interaction of aluminum with bovine brain calmodulin. The aim of the investigation was to detect aluminum-induced changes in calmodulin conformation. We found that aluminum binds stoichiometrically to calmodulin and enhances the hydrophobic surface domain relative to that exposed in the presence of calcium. These changes are reflected in the inhibition of the calmodulin-dependent activity of 3',5'-cyclic nucleotide phosphodiesterase. Aluminum-induced conformational changes in calmodulin are examined by fluorescence spectroscopy and circular dichroic spectrophotometry.

Materials and Methods

Calmodulin preparation. Calmodulin was prepared from bovine brain acetone powder as described previously [19]. However, to increase the purity of the isolated product, the protein-loaded affinity column was washed with a buffer containing 20 mM Mes/NaOH, pH 7, 500 mM NaCl and 1 mM mercaptoethanol. Subsequently, the calmodulin was eluted with the same buffer containing 10 mM EGTA. $CaCl_2$ was immediately added to the calmodulin fraction collected to give a final concentration of 15 mM. Following dialysis against 10 mM ammonium carbonate and distilled water [19], the calmodulin fraction was further subjected to electrodialysis, applying a current between 5 and 10 mA for about 4 h. The final solution was lyophilized and the product was stored in a desiccator at −40°C. This product was reconstituted in deionized water and dialyzed first against 100 μM EDTA, then exhaustively against deionized water, and finally against deionized buffer containing Chelex-100 resin. This material stimulated 3',5'-cyclic nucleotide phosphodiesterase, had an ultraviolet absorption spectrum typical to that of calmodulin [19], and migrated as a single band on SDS-polyacrylamide gels (15%) as visualized by the silver staining technique [20]. Protein concentrations were determined by a modification [21] of the method of Lowry et al. [42], or by measuring an absorption spectrum with a Gilford spectrophotometer, model 2400; the molar extinction coefficient is 3300 $M^{-1} \cdot cm^{-1}$, at 277 nm [13]. Calmodulin isolated as described above contained less than 10^{-8} M calcium, magnesium, manganese and aluminun. This quantitative analysis was performed with a Jarrell-Ash plasma emission spectrometer, model 955 Atomcomp, and a Varian atomic absorption spectrophotometer, model 1475.

Materials. Bovine brain acetone powder, Tris, Mes, PSMF, EDTA, EGTA, Mops, 5'-nucleotidase and 3',5'-cyclic nucleotide phosphodiesterase were purchased from Sigma Chemical Co. (St. Louis, MO). [8-^{3}H]Guanosine 3',5'-cyclic phosphate was obtained from New England Nuclear Corp. (Boston, MA). $AlCl_3$, $CaCl_2$ and NaCl were obtained from Mallinckrodt Science Products (St. Louis, MO). Chelex-100, AG 1X-8, and Affigel phenothiazine were purchased from Bio-Rad Laboratories (Richmond, CA). The sodium salt of ANS was obtained from K & K Laboratories (Plainview, NY). (+)-10-Camphorsulfonic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of the highest quality available.

All glassware and quartz cuvettes were washed in concentrated nitric acid. Buffer solutions were prepared in double, glass-distilled deionized water and passed over columns (2 × 30 cm) of Chelex-100. Metal stock solutions of calcium and aluminum chloride were freshly prepared in decontaminated buffers. Plasma emission and atomic absorption spectroscopy indicated buffer solutions typically to contain less than 10^{-8} M calcium, magnesium, manganese and aluminum.

Phosphodiesterase assay. Hydrolysis of cyclic GMP by 3',5'-cyclic nucleotide phosphodiesterase was determined by the procedure described by Wolff et al. [11], with the following modifications. 300-μl reaction volumes containing 25 μM

carrier-labelled cGMP and 5.5 μM calmodulin in 10 mM Tris-HCl, pH 6.5, were constructed in polyethylene containers. An aliquot of activator-deficient 3',5'-cyclic nucleotide phosphodiesterase was added to start the reaction; reaction times were allowed to vary between 15 and 30 min, at 37°C. The reaction was stopped by boiling the sample for 2 min. After the solution was cooled to ambient temperature, 5'-nucleotidase was added and the sample was incubated for 20 min at 37°C. This reaction was stopped by adding a 1-ml aliquot of AG 1X-8 resin, diluted 1 : 2 (v/v) in 50% isopropanol. The reaction vials were centrifuged and the supernatants analyzed in a Beckman scintillation counter, model LS 7000, to determine the amount of hydrolysis.

Circular dichroism experiments. Some CD spectra were recorded at ambient temperature on a Jasco spectropolarimeter, model ORD/UV/CD-5, modified by Sproul Scientific Instruments (Boulder Creek, CA). For purposes of digital signal processing, CD spectra were also recorded on a Jasco automatic recording spectropolarimeter, model J-40 C, interfaced with a Data General Nova-3 processor and a Tracor-Northern digital signal analyzer, model TN-1500. Both instruments were calibrated to a molar ellipticity of $[\theta] = +7260$ deg · cm^2 · dmol^{-1}, at 290.5 nm, for a 0.1% aqueous solution of (+)-10-camphorsulfonic acid [22]. Samples of calmodulin were prepared from the lyophilized protein reconstituted as described in Materials and Methods, in deionized 10 mM Tris-HCl, adjusted to pH 6.5 with concentrated HCl. CD spectra were obtained from samples in rectangular quartz cells of 1-cm path length. Calmodulin solutions of 3-ml volume were titrated with 3–10-μl aliquots of calcium or aluminum chloride salts from stock solutions.

Mean residue ellipticities of calmodulin, $[\theta]$, were calculated from the relation $[\theta] = \theta_{obs} M/100\,lc$, where θ_{obs} is the observed elliptical value, M is the mean residue molecular weight, taken to be 117 [11], l is the optical path length in decimeters, and c is the protein concentration in g/ml. The relative structural contents of calmodulin were calculated by the procedure of Chen et al. [23]. The helical content was estimated from the relationship % α-helix $= -([\theta]_{222} + 2340)/303$, where $[\theta]_{222}$ is the mean residue ellipticity at 222 nm. Relative contents of α-helix were also estimated according to the procedure of Greenfield and Fasman [24] for the purpose of comparing the measured values with those reported in the literature. The Hill coefficient, h, was determined from the expression $R_x = 81^{1/h}$, where the cooperativity index, R_x, has been derived from experimental data [25].

Fluorescence measurements. Fluorescence intensity measurements were performed on a Perkin-Elmer spectrofluorimeter, model MPF-44A, equipped with a differential corrected spectra unit. The excitation and emission wavelengths for tyrosine fluorescence were 280 ± 2 and 320 ± 5 nm; for ANS fluorescence studies 360 ± 4 and 490 ± 4 nm, respectively. For measurements of tyrosine fluorescence, calmodulin was dissolved in 100 mM NaCl, and maintained at pH 6.5 with NaOH. For ANS fluorescence measurements, the sample was prepared in 10 mM Mops buffer, at a 10 μM concentration, adjusted to pH 6.5 with NaOH. ANS concentration of 2 μM. A cuvette of 1 cm optical pathlength was used. The spectra were recorded at room temperature. The absorbance at the excitation and emission wavelengths was less than 0.05.

Metal ion titration. Maximum fluorescence is defined as fluorescence intensity of tyrosine emission of calmodulin, in the absence of EGTA and metal ions. Upon addition of 100 μM EGTA, tyrosine fluorescence was quenched, and upon subsequent addition of 1–2-μl aliquots of metal stock solution to the 2 ml sample in the cuvette, a fluorescence increase of tyrosine emission was observed. Data for tyrosine fluorescence, in the presence of metal ions, are expressed as percent of maximum fluorescence intensity. The ANS fluorescence intensity of a calmodulin solution, in the absence of metal ions, was considered as the initial fluorescence intensity value. This initial value increased upon addition of 1–2-μl aliquots of metal stock solution. Data for ANS fluorescence, in the presence of metal ions, are expressed as the relative increase of the initial fluorescence intensity value. A value of zero is defined as the fluorescence value with protein absent.

Equilibrium dialysis. For equilibrium dialysis calmodulin samples were reconstituted in 10 mM Tris-HCl, pH 6.5. Sample volumes of 2 ml were

put in dialysis membranes having a molecular weight cutoff at 3400 and dialyzed against 100-ml volumes of various aluminum concentrations in the same buffer for 24 h at room temparature. Aluminum concentrations were determined according to the following procedure. 5-μl aliquots of concentrated nitric acid were added to 1 ml sample volumes. After 10 min, 1 ml of 1 M acetate buffer, pH 6.0, was added, followed by 0.25 ml of 0.01 mg/ml Eriochrome cyanine R in water. After 20 min the absorbance was measured at 535 nm. Standards were prepared by dilution of a 1000 μg/ml aluminum atomic absorption standard solution (Aldrich Chemical Co., Milwaukee, WI). The value of the dye absorbance was shown to be accurate between 0.1 and 1 ppm aluminum, as compared with the value obtained from direct measurements of aluminum in a plasma emission spectrometer. Interference caused by the presence of protein was accounted for.

Results

CD studies of aluminum-induced changes in calmodulin

The ultraviolet circular dichroism spectra of calmodulin show increasing negative ellipticities upon titration of calcium to the protein solution. Minima of negative ellipticity appear at 222 and 207 nm (Fig. 1A), consistent with CD spectra reported for calmodulin [11]. From these spectral features relative amounts of secondary structures can be estimated [23]. We find that a protein structure containing 37% α-helix, 11% β-sheet and 52% random coil generates a CD spectrum which fits best to that observed for metal-free calmodulin. These values agree with those reported previously; the helical content has been found to vary between 28 and 45% for metal-free calmodulin and to increase by 10–15% upon calcium binding to calmodulin, at a molar ratio of 4:1 [3,6,26]. Applying the Greenfield and Fasman procedure [24], a helix content of 31% can be calculated from our data for the native protein, while Wolff et al. [11] determined a value of 28%.

Analysis of the CD spectra of calmodulin titrated with aluminum (Fig. 1B) indicates a spectral shift towards decreasing values of negative ellipticity, contrary to the shift observed upon

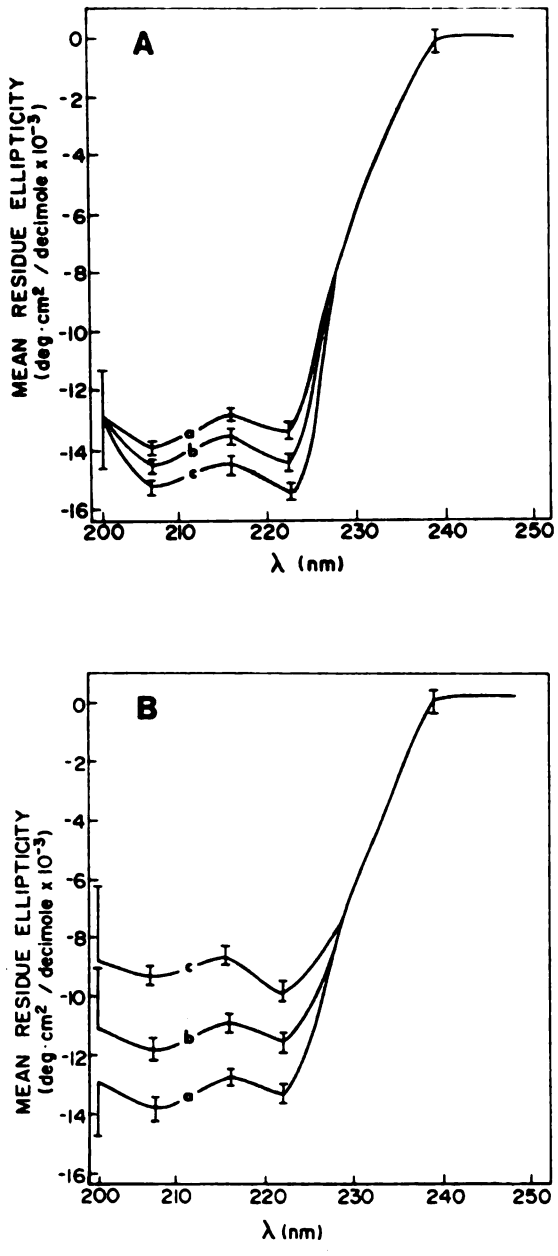

Fig. 1. Changes in the CD spectrum of bovine brain calmodulin in the presence of (A) calcium and (B) aluminum. Calmodulin was reconstituted in metal-free 10 mM Tris-HCl buffer, pH 6.5, at a final concentration of 10 μM. A, $CaCl_2$ was titrated to a final concentration of (a) 0, (b) 20 and (c) 40 μM. B, $AlCl_3$ was titrated to a final concentration of (a) 0, (b) 20 and (c) 50 μM. The spectra were invariant with respect to time. Error bars indicate the actual noise levels observed during multiple scans.

application of increasing calcium concentrations. The aluminium-induced spectral shift even takes place in the presence of saturating levels of calcium (data not shown). Experimentally determined values of $[\theta]_{222}$ and calculated values of the helix content are plotted vs. the metal concentration (Fig. 2). Relative to values derived for metal-free calmodulin, the helical content increased by 17% upon calcium binding, whereas it decreased by 30% upon binding of aluminum to calmodulin at a molar ratio of 4 : 1. The aluminum-induced change even occurred in the presence of 100 μM $CaCl_2$ (data not shown). From respective metal titration curves, midpoint ratios (mol/mol) of 2 : 1 for [calcium]/[calmodulin] and 2.5 : 1 for [aluminum]/[calmodulin] can be derived. Additional information is listed in Table I. CD spectra for calcium titration at pH 7.5 were identical to those measured at pH 6.5 (data not shown). From plots of $[\theta]_{222}$ vs. metal concentration, Hill coefficients of 1.54 and 1.63 can be calculated for calcium and aluminum binding to calmodulin — these values

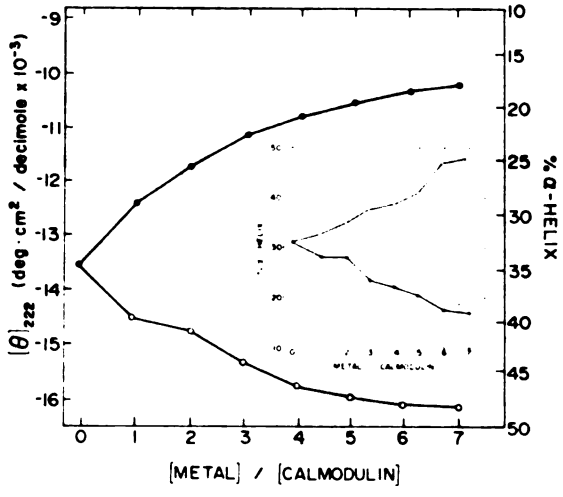

Fig. 2. Effects of metal titration on the mean residue ellipticity, $[\theta]$, at 222 nm, and the helical content of bovine brain calmodulin. Conditions were the same as those described in the legend to Fig. 1. Titration was performed with $CaCl_2$ (○) or $AlCl_3$ (●). Mean values for at least four separate calmodulin preparations are plotted; error 5%. Helical content is estimated from the relationship: % helix = $-([\theta]_{222} + 2340)/303$. Inset: Computed helical content according to the procedure of Chen et al. [23], taking into account the presence of β-sheets and random coils.

TABLE I

STRUCTURAL CHANGES IN BOVINE BRAIN CALMODULIN INDUCED BY METAL BINDING

Results were calculated by the method of Chen et al. [23].

[metal]/[calmodulin] (mol/mol)	Metal	% helix	% sheet	% coil
0	-	37	11	52
2	Ca^{2+}	40	9	50
4	Ca^{2+}	44	9	47
2	Al^{3+}	28	7	65
5	Al^{3+}	22	5	73

are indicative of positive cooperativity for the changes observed.

Aluminum binding increases the hydrophobic surface of calmodulin

Titration of calmodulin with aluminum or calcium induces the exposure of a hydrophobic surface on the protein, as evidenced by partition studies which employed the fluorescent hydrophobic probe, ANS. This molecule has been used to investigate the expression of hydrophobic surface domains on calmodulin by calcium binding [14]. Aluminum enhanced the ANS fluorescence intensity more effectively than did calcium in the presence of calmodulin (Fig. 3). The fluorescence intensity reached saturation at an [aluminum]/[calmodulin] ratio of 3 : 1 (mol/mol). The aluminum-induced fluorescence changes is five times that produced by calcium and remained at this value even in the presence of 120 μM $CaCl_2$ (data not shown). This aluminum-induced change can be characterized by a Hill coefficient of 1.86, indicative of positive cooperativity.

Intrinsic fluorescence of calmodulin

Bovine brain calmodulin harbors two tyrosyl residues, viz., Tyr 99 and Tyr 138, which are located at the calcium-binding sites III and IV, respectively [12]. Tryptophan residues are lacking. Therefore, measurements of tyrosine fluorescence can be performed to investigate induced conformational changes of the protein.

The aluminum-induced increase in tyrosine fluorescence intensity of calmodulin saturated at a

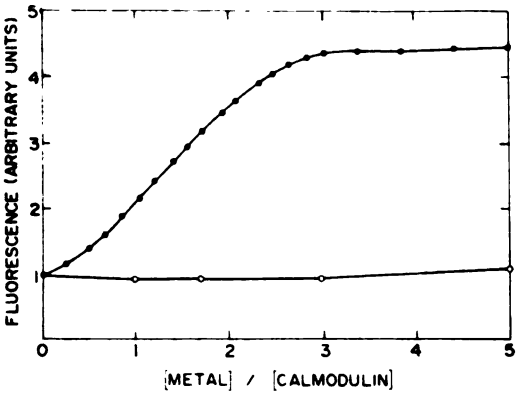

Fig. 3. Fluorescence of 8-anilino-1-naphthalene sulfonic acid in the presence of bovine brain calmodulin and increasing metal content. $CaCl_2$ (○) or $AlCl_3$ (●) was titrated into a solution of 10 μM calmodulin in 10 mM MOPS buffer, pH 6.5, as described in Materials and Methods. The fluorescent probe was present at 2 μM. The data shown are from four separate calmodulin preparations and represent mean values within 5% error. The zero fluorescence value is defined as that of ANS in the absence of calmodulin

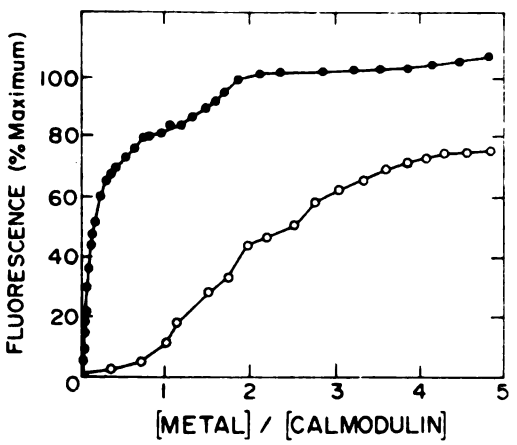

Fig. 4. Tyrosine fluorescence of bovine brain calmodulin as a function of metal concentration. A 9 μM concentration of calmodulin was prepared in 100 mM NaCl, pH 6.5. After addition of EGTA to give a final concentration of 100 μM, the protein solution was titrated with $CaCl_2$ (○) or $AlCl_3$ (●). 100% fluorescence intensity corresponds to the intensity emitted from calmodulin prior to EGTA and metal additions. Excitation was performed at 280 nm, emission was recorded at 320 nm.

molar ratio of 2 : 1. The first phase of fluorescence enhancement occurred at a ratio of 1 : 1 [aluminum]/[calmodulin]; this initial phase was characterized by a large and steep increase (Fig. 4). The steepness of the increase is reflected in a Hill coefficient of 1.2. The aluminum concentration at which a 50% increase in tyrosine fluorescence occurred is al least one order of magnitude lower than the respective calcium concentration (Fig. 4). The initial phase of aluminum-induced fluorescence enhancement probably results from binding of the metal near or at Tyr 138, since this residue has been found to be most sensitive to metal-induced conformational changes [27]. This interpretation is also consistent with results from NMR experiments demonstrating that binding of one calcium ion per calmodulin shifted the resonances of Tyr 138 [28,29]. The aluminum-induced changes in tyrosine fluorescence may also be a consequence of long-range conformational changes. The steepness of the aluminum-induced response indicates that at least the first two aluminum ions are bound to calmodulin in a positive cooperative manner. This observation is consistent with results derived from kinetic data of calcium binding to the protein [30].

The dequenching experiments were carried out in the presence of a chelator. This chelator, EGTA, does not bind significantly to calmodulin [27]. Furthermore, the removal of calcium by excess chelator from calmodulin was independent of chelator type and concentration [30]. Binding of EGTA to calcium-binding proteins has been described [12]. Stability constants, expressed as log K, for aluminum-EGTA and calcium-EGTA, at the pH used, are 3.97 and 3.79, respectively [31]. Therefore, the observed changes are not a result of differences in metal-EGTA interactions.

Extrapolation of the data presented in Fig. 4 indicates a value for the dissociation constant of about 0.1 μM, for the first mol of aluminum bound. This compares well with similarly obtained values for aluminum dissociation constants derived from data obtained from CD and ANS experiments, viz., 0.2 and 0.4 μM, respectively. Dissociation constants for calcium binding derived from these data correspond to published values of about 1 μM [3]. Considering the cooperative character of metal binding, we confined ourselves to estimating the dissociation constant for the first

mol of aluminum bound to calmodulin. These spectroscopically derived dissociation constants for aluminum binding agree with a value of 0.4 μM derived from equilibrium dialysis studies. Results from the latter studies also show the existence of three metal-binding sites on the protein (Fig. 5). We believe that the dissociation constant, ranging from 0.1 to 0.4 μM, is indicative of the order of magnitude of the high-affinity binding constant for metal binding. Clearly, a thermodynamic analysis is necessary for a detailed evaluation of dissociation constants.

Aluminum inhibits phosphodiesterase

Calcium-dependent calmodulin activation of 3',5'-cyclic nucleotide phosphodiesterase is a prime example of the modulatory role for calmodulin [6,32]. Under our experimental conditions, addition of calcium doubled the initial enzymatic activity from 0.28 to 0.61 nmol cyclic GMP hydrolyzed/ml per min. Elevated activation has been shown to depend on the presence of imidazole and millimolar concentrations of Mg^{2+} [6,26]. Titration of aluminum into the assay system containing 25 μM calcium lowered the hydrolyzing activity to levels equivalent to basal activity in the absence of calcium. The aluminum concentration which inhibited the enzymatic activity by 50% is calculated to be 15 μM, representing a molar ratio of 3 : 1 for [aluminum]/[calmodulin]. Results from separate experiments indicated that in the absence of Ca^{2+} aluminum did not interfere with the basal phosphodiesterase activity. Aluminum appeared to interact with calmodulin rather than the enzymatic protein (Fig. 6).

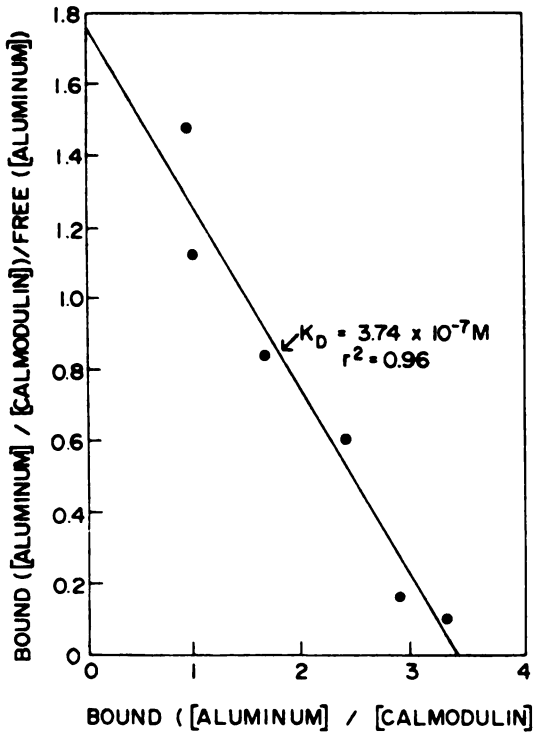

Fig. 5. Binding data from equilibrium dialysis experiments, presented as a Scatchard plot. 5 μM calmodulin was dialyzed against various amounts of $AlCl_3$ in 10 mM Tris-HCl, pH 6.5, for 24 h. 1-ml aliquots of solution, each from inside and outside of the dialysis bag, were assayed for aluminum as described in Materials and Methods. r^2 is the correlation coefficient.

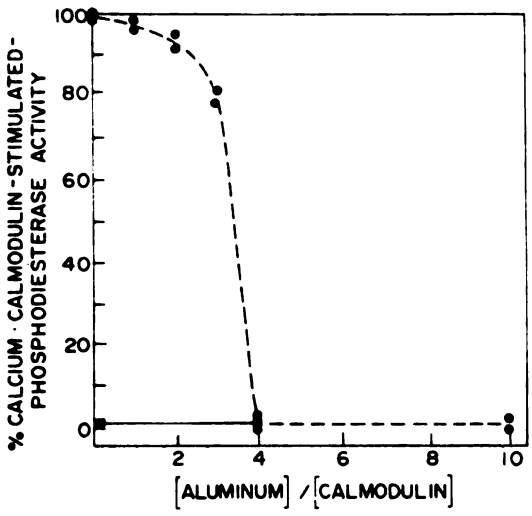

Fig. 6. Inhibition of calcium-calmodulin-stimulated 3',5'-cyclic nucleotide phosphodiesterase activity by aluminum. The enzyme was incubated with 25 μM cyclic GMP, 5.5 μM calmodulin and 25 μM $CaCl_2$, in 10 mM Tris-HCl, pH 6.5. Incubation times varied between 15 and 30 min. 100% inhibition equals the basal enzyme activity observed with calcium absent. The enzymatic activity was determined as described in Materials and Methods. The enzymatic activity was measured in the presence (●) or absence of calcium (■).

Discussion

The results presented in this study show that aluminum binds stoichiometrically to bovine brain

calmodulin. This indicates that aluminum ions are bound to specific sites on calmodulin. Considering the difference in charge and that of crystal or hydrated radii [33], it is doubtful whether the aluminum-binding sites are identical to those for calcium. The binding regions for aluminum may overlap those for calcium and the curvature of the respective binding loops may also vary. There seem to exist two 'high-affinity' binding sites, as demonstrated, for example, in our fluorescence experiments. Our estimates indicate that the 'high-affinity' binding for aluminum to calmodulin is about one order of magnitude stronger than that of calcium to its comparable site. Similar to the interaction of Ca^{2+} to calmodulin, two aluminum ions per protein are sufficient to induce the major structural change as evidenced by CD and ANS fluorescence studies. However, a molar ratio of 4:1 is required to block the phosphodiesterase activity maximally.

Considering the pronounced aluminum-induced conformational changes, at least for the first two ions, we have to consider the possibility that the higher binding constant, relative to that for calcium, results from a positive entropy contribution. A major factor for such a positive change is probably the release of coordinated water molecules around the metal ion upon complex formation with the protein. Both Al^{3+} and Ca^{2+} have a primary coordination number of 6, which represents the effective hydration number of the innermost hydration shell [34]. It has been suggested that there exists a single water molecule at the high-affinity binding site of troponin C, a protein which is closely related to calmodulin [35]. Therefore, five coordinated water molecules are to be released for each calcium ion bound to the protein. The entropy of calcium binding to the troponin sites 1 and 2 has been measured to be about 15 e.u. [36]. The strength of these coordinated bonds, responsible for ion/water interactions, is appreciably higher for Al^{3+}, which has an hydration enthalpy of 1 144 kcal/mol of ion, as compared to a value of 399 kcal/mol for Ca^{2+} [37]. Moreover, the value of the formal charge over ionic radius and the intermediate electronegativity are both a factor of 2 higher for Al^{3+} relative to Ca^{2+} [37]. Also the number of water molecules in the outer hydration shells of Al^{3+} is significantly higher than that for Ca^{2+} [34]. Considering these physical data, interactions of aluminum ions with calmodulin are expected to differ appreciably from those of Ca^{2+}. In particular, the solvent structure around protein ligands should depend on the respective coordinated metal ion. As far as calcium is concerned, it is known that this ion appears to be coordinated to oxygen ligands and mobility of the ion is restricted considerably when bound to the protein [38,39]. Al^{3+} also forms stable complexes with electronegative ligands such as oxygen and nitrogen. In contrast to calcium, the higher electronegativity of Al^{3+} would result in an increased covalent character of the coordinate bond established. Consistent with our observations, the stability of the aluminum-calmodulin complex would therefore be increased as compared to that of the calcium complex.

Aluminum-induced conformational changes in calmodulin are also documented by our CD studies. While Ca^{2+}, and the spatially isomorphous lanthanide, Tb^{3+}, promote helix formation [27], the helix content is decreased by about 30% when Al^{3+} is present with calmodulin at a molar ratio of 4:1. Charge differences per se cannot account for this metal type-dependent change in helix structure since the trivalent ion, Tb^{3+}, promotes helical formation, just as Ca^{2+}. Rather, the unique physicochemical characteristics of the highly solvated aluminum ion, as discussed above, are hypothesized to be responsible for the observed structural alterations. It is thought that upon binding of Ca^{2+} to calmodulin negative charges on the protein will be neutralized. This in turn results in weakening of constraining forces, thus permitting the formation of additional helical elements. Finally, a more compact calcium-calmodulin complex emerges [29]. As far as interactions of Al^{3+} with calmodulin are concerned, their molecular origin and significance are presently unclear, and further experiments are necessary. What can be stated is that under our experimental conditions mononuclear, hydrated aluminum species are present, as opposed to polynuclear species existing at higher pH values and elevated aluminum concentrations [40]. As to thermodynamic changes, application of aluminum ions to calmodulin leads to a helix-coil transition, which is accompanied by a strong enhancement of the protein's hydro-

phobic surface domains. Such behavior is to be expected for a randomly coiled polypeptide with an increased portion of hydrophobic components. Simply stated, the hydrated aluminum ions promote an open, solvent-rich, disordered polypeptide region whereas calcium ions promote a peptide environment where intramolecular interactions between adjacent peptide elements are favored. The importance of ion-dependent changes in solvent structure is further exemplified by qualitative experiments showing that calmodulin aggregates above a molar ratio of 10:1 for [aluminum]/[calmodulin], in contrast to calcium-calmodulin complexes.

In summary, it appears that aluminum binding to specific regions of calmodulin results in local structural changes which, in turn, have profound consequences for the relative motion of distinct, internal structural domains [41]. As a result, the protein's flexibility and its ability to interact with various proteins is impaired. These kinds of changes in calmodulin may explain why the aluminum-calmodulin complex lost at least part of its regulatory character. This complex may thus be a key lesion that occurs in the broadly defined syndrome of aluminum toxicity, if viewed in terms of lost regulatory capacity.

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Chapter 4

Calmodulin-Dependent Formation of Membrane Potential in Barley Root Plasma Membrane Vesicles: A Biochemical Model of Aluminum Toxicity in Plants

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Calmodulin-dependent formation of membrane potential in barley root plasma membrane vesicles: A biochemical model of aluminum toxicity in plants

Neal Siegel and Alfred Haug

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Micromolar concentrations of aluminum ions interfere with calmodulin-stimulated, membrane-bound ATPase activity which plays a role in the maintenance of the transmembrane potential of plasma membrane-enriched vesicles isolated from barley roots. Calmodulin appears to be the major target for aluminum interaction resulting in pronounced changes in the exposure of a large, hydrophobic surface on this protein as determined with a fluorescent, hydrophobic surface probe. At a molar ratio of 3:1 [aluminum]/[calmodulin], the calmodulin-stimulated enzymatic activity, probably associated with a Ca^{2+} + Mg^{2+}–ATPase, is about 95% inhibited. Aluminum-induced changes in calmodulin structure are reflected in reduced formation of the membrane potential when assayed with a fluorescent potential probe, oxonol VI. We hypothesize that the aluminum-calmodulin complex represents a primary lesion in toxic responses of plants to this metal.

Additional key words – Ca^{2+} + Mg^{2+}-ATPase activity.

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Introduction

Following mobilization at acidic pH, aluminum ions are potent, toxic agents to plants. Elevated soil aluminum levels were shown to impair root elongation and to interfere with the uptake, distribution and use of calcium, magnesium, phosphorus and other essential minerals (Foy et al. 1978). Elevated tissue aluminum levels were reported to cause chloroplast membrane degeneration and to decrease CO_2 fixation in spinach (Hampp and Schnabl 1975). Upon application of aluminum to barley roots, plasma membrane degeneration seems to be an early indication that aluminum is present at toxic levels (Hecht-Buchholz and Foy 1981). The mode of interaction of aluminum with living tissue is unknown although it has been observed that aluminum binds to ATP, which in turn impairs yeast hexokinase (Viola et al. 1980). Aluminum can also bind to DNA and impair genetic expression (Foy et al. 1978). Among several varieties of wheat, barley and soybeans, aluminum tolerance seems to be associated with resistance against aluminum-induced calcium deficiency or reduced calcium transport, and has been shown in barley to be linked to a single, dominant gene (Foy et al. 1978).

As a result of aluminum-induced changes in calcium uptake and utilization, biochemical systems dependent on calcium for regulation may be targets of aluminum ions. In plant as in animal cells, free calcium levels are strictly regulated and do not exceed micromolar concentrations (Clarkson and Hanson 1980). Activation of key, calcium-regulated processes in many instances involves calmodulin. This small (MW = 17 000), acidic, calcium-dependent regulatory protein responds to transient increases in intracellular calcium levels and has been shown to regulate Ca^{2+}-ATPase activity in plants (Caldwell and Haug 1981a, Dieter and Marmé 1980, 1981). In addition, aluminum ions have been shown to interact with calmodulin in such a way that the

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protein's regulatory capacity diminishes and becomes lost with increasing molar ratios of [aluminum]/[calmodulin] (Siegel and Haug 1983).

In the present study, we intend to demonstrate that the calmodulin-regulated, Ca^{2+}- and Mg^{2+}-dependent ATPase activity in barley root plasma membranes is electrogenic and interfered with by aluminum. Effects of various metals, pH, calmodulin, ionophores and aluminum on the electrogenic activity are described. These data will be dicussed in terms of lost regulatory capacity by calmodulin resulting in the failure of the plasma membrane to maintain an elctrical potential necessary for proper cell maintenance.

Abbreviations – ANS, 8-anilino-1-naphthalene sulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DTT, dithiothreitol; MES, morpholinoethane sulfonic acid; MOPS, 4-morpholinopropane sulfonic acid; oxonol VI, propyl oxonol; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulfate.

Materials and methods

Calmodulin preparation

Calmodulin was prepared from bovine brain acetone powder as previously described (Caldwell and Haug 1981b). However, to increase purity of the yield, the protein-loaded affinity column was washed with a buffer containing 500 m*M* NaCl (Siegel and Haug 1983). For our experiments we used bovine brain calmodulin since its structure is well known (Klee et al. 1980) and because this protein stimulates barley root plasma membrane ATPase activity in the identical way to calmodulin extracted from barley (Caldwell and Haug 1981a).

Chemicals

Bovine brain acetone powder, DTT, EDTA, MES MOPS, PMSF, sodium-ATP and Tris were purchased form Sigma Chemical Co. (St. Louis, MO). $AlCl_3$ and $CaCl_2$ were purchased from Mallinckrodt Science Products (St. Louis, MO). Affigelphenothiazine and Chelex-100 were purchased from Bio-Rad Laboratories (Richmond, CA). The sodium salt of ANS was obtained from K&K Laboratories (Plainview, NY). A-23187 and nigericin were obtained from Calbiochem-Behring Corp. (La Jolla, CA). Chlorpromazine HCl and trifluoperazine HCl were obtained from Smith, Kline and French Labs. (Philadelphia, PA). Oxonol VI was purchased from Molecular Probes, Inc. (Junction City, OR). All other chemicals were of the highest purity commercially available.

Plant material and growing conditions

Barley seeds (*Hordeum vulgare* L. cv. Conquest) were germinated and grown in the dark at 16°C over aerated solutions of 0.25 m*M* $CaSO_4$, adjusted to pH 5.0 with H_2SO_4. The growth medium was changed daily. Six days after imbibition the primary roots were washed in chilled, distilled water and excised. All handling of the material was done at 4°C.

Preparation media

Homogenizing medium: 0.25 *M* sucrose, 3 m*M* EDTA, 1 m*M* PMSF, and 1 m*M* Na_2-ATP were prepared in 25 m*M* Tris-MES buffer adjusted to pH 7.2. Sucrose gradient: The discontinuous gradient was prepared from 34% and 40% (w/w) sucrose solutions in 1 m*M* Tris-MES, pH 7.2. Wash solution for the isolated membranes: 0.25 *M* sucrose in 1 m*M* Tris-MES buffer, pH 6.5.

Membrane isolation

A plasma membrane enriched microsome fraction was isolated according to the method of Nagahashi et al. (1978), as modified by Caldwell and Haug (1980) with minor modifications, using the following scheme:

1. Roots (approximately 200 g) were homogenized in 3 ml of homogenizing solution per gram of tissue by hand using a ceramic mortar and pestle without the addition of any abrasive material.
2. Filtration through miracloth.
3. Centrifugation 1 300 *g*, 15 min, discard pellet.
4. Centrifugation 80 000 *g*, 30 min, supernatant discarded.
5. Pellet resuspended in wash solution.
6. Resuspended membranes loaded onto discontinuous gradient.
7. Centrifugation 80 000 *g*, 2 h.
8. Membrane at interface (34%/40%) removed.
9. Interface resuspended in wash solution.
10. Centrifugation 80 000 *g*, 30 min.
11. Resuspend pellet in wash solution at a concentration of 0.5 mg vesicle protein ml^{-1}.
12. Membranes were stored for a maximum of 24 h on ice in a cold room kept at 4°C.

Determination of membrane potential

Fluorescence measurements of oxonol VI were made in a 2 ml reaction volume containing various concentrations of divalent cations, calmodulin, aluminum or ionophores at the desired pH in the wash medium. Vesicles were diluted to 50 µg vesicle protein ml^{-1}. All experiments were carried out at 16°C. After a 15 min preincubation of the vesicles in the adjusted wash medium, oxonol VI was added from a concentrated stock solution to a final concentration of 0.3 µ*M*. The solution was then transferred to a quartz cuvette with a 10 mm optical path length which was placed into a thermostatted holder of a fluorimeter (Jen and Haug 1981). The excitation and emission wavelengths were

580 nm and 640 nm, respectively. Light scattering was negligible.

A baseline was established by adjusting the suppression current of the picoammeter until the value had stabilized. An aliquot (20 μl) of 100 m*M* Tris-ATP (Hodges and Leonard 1974) was injected from a microsyringe into the sample to begin the reaction. Fluorescence signal changes were monitored and recorded for up to 20 min. First order rate constants, k, were calculated from the relationship $F = F_0 \cdot \exp(-kt)$, using a Tektronix 4051 computer and non-linear, least-squares equation fitting programming. F and F_0 are the fluorescence intensities at times t and t = 0, respectively.

ANS fluorescence measurements

Fluorescence intensity measurements were performed on a Perkin-Elmer spectrofluorimeter, model MPF-44A, equipped with a differential corrected spectral unit. The excitation and emission wavelengths were set at 360 ± 4 nm and 490 ± 4 nm, respectively. Calmodulin was prepared in 10 m*M* MOPS, pH 6.5, at a final concentration of 10 μ*M*. ANS was added from a concentrated stock solution to a final concentration of 2 μ*M*. A quartz cuvette of 10 mm optical pathlength was used. The ANS fluorescence intensity of a calmodulin solution in the absence of metal ions was considered to be the initial fluorescence intensity value. Data for ANS fluorescence in the presence of metal ions are expressed as the relative increase of the initial fluorescence intensity value. A value of zero is defined as the fluorescence of the solution without calmodulin present.

Protein analysis

Protein was quantified with bovine serum albumin as a standard (Wang and Smith 1975). Calmodulin concentrations were also adjusted spectrophotometrically using a value for the molar extinction coefficient of 3 300 M^{-1} cm^{-1} at 277 nm (Crouch and Klee 1980).

Removal of contaminating metals

All glassware and quartz cuvettes were washed with concentrated nitric acid. Buffer solutions were prepared in double, glass-distilled, deionized water and passed over columns (2 cm × 30 cm) of Chelex-100 resin. Metal stock solutions were freshly prepared in metal-decontaminated buffers. Plasma emission and atomic absorption spectroscopy indicated that the buffer solutions and the Tris-ATP preparations typically contained less than 10^{-6} *M* calcium, magnesium, and aluminum. This quantitative analysis was performed on a Jarrell-Ash plasma emission spectrometer, model 955 Atomcomp, and a Varian atomic absorption spectrophotometer, model 1475.

Data analysis

The presented data are means of at least three independent experiments ± standard deviation except when otherwise noted.

Results

Oxonol VI as a probe of transmembrane potential

The fluorescent oxonol probe is voltage sensitive and is therefore useful for evaluating changes in transmembrane potentials (Beeler et al. 1981, Smith et al. 1981). Subsequent redistribution of the dye in beef-heart submitochondrial vesicles was shown to produce a fluorescence intensity loss upon Mg^{2+}-ATP stimulation of ATPase activity involved in establishing an electrochemical gradient across these membranes. Our results derived from time-dependent fluorescence signal changes are qualitatively consistent with these data. The fluorescence decay curves from our experiments were best fit by a single exponential function which is consistent with data from the literature and is a consequence of the low affinity of oxonol VI for phospholipid membranes (Smith et al. 1981). The direction of fluorophore distribution is across membranes with an inside-positive potential as a result of this probe's delocalized negative charge (Beeler et al. 1981).

Addition of ATP to the vesicle suspension activated the Ca^{2+}-ATPase resulting in a sustained, fluorescence intensity decrease (Fig. 1). As a control, vesicles were boiled for 2 min; immediately upon addition of ATP, the fluorescence intensity of oxonol increased slightly, but quickly reached a new steady-state. Upon preincubation of vesicles in 0.01% SDS or Triton X-100 (re-

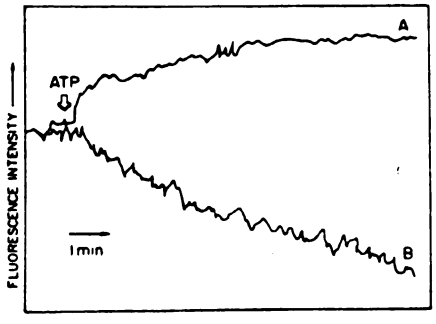

Fig. 1. Changes in fluorescence intensity of oxonol VI in the presence of membrane vesicles from barley roots. Traces show responses of (A) vesicles which were boiled for 2 min, or (B) vesicles which were untreated. Conditions for assay: 0.05 mg vesicle protein ml^{-1}, 0.25 *M* sucrose, 1 m*M* Tris-MES (pH 5.5), 1 m*M* $CaCl_2$, 0.3 μ*M* oxonol VI, 16°C. The vesicles were allowed to pre-incubate until a stable value for the fluorescence signal was established. Tris-ATP was then added to 1 m*M* (arrow).

sults not shown), the fluorescence signal was indistinguishable from that of the dye in the absence of vesicles. Analysis of the fluorescence signal after ATP activation of the vesicle suspension indicated that, in some cases, two components were present. The first, fast component became negligible within 1 to 2 min and was considered as resulting from turbulence in the assay solution after mixing. First order rate constants were calculated from all traces after 5 min to avoid interference.

pH dependence of the Ca^{2+}- and Mg^{2+}-dependent ATPase activity and its relation to the development of the transmembrane potential

Ca^{2+}- and Mg^{2+}-ATPase activities were independently activated by the respective cations and the effect of each on transmembrane potential development compared (Fig. 2). The observed pH response profiles for both activities are consistent with those results previously derived for the high-affinity Ca^{2+}- and Mg^{2+}-ATP hydrolyzing activities of the ATPase in these same membrane vesicles (Caldwell and Haug 1980). Calcium at 1 m*M* sustained a high rate of potential development at pH 5, but dropped below the value for 1 m*M* Mg^{2+} at increasing pH values. Above pH 5.5, Mg^{2+} sustained a fairly constant rate between 0.12 min^{-1} and 0.11 min^{-1}, whereas the rate sustained by Ca^{2+} decreased 72% from 0.11 min^{-1} to 0.03 min^{-1} in the same pH range. Under these conditions the concentration of free calcium is higher than that found intracellularly in vivo. It is for the purpose of comparison with previous enzymatic studies that these concentrations of the divalent cations were used (Caldwell and Haug 1980). The decline in the ATPase-controlled membrane potential change may be related to the effects of positively charged enzyme-substrate complexes at lower pH for both Ca^{2+}- and Mg^{2+}-ATPase activities, whereas the rates measured at elevated pH values (above pH 6.0) may be the result of deprotonation leading to charge neutralization of the enzyme-substrate complex. The pH dependence of the development of the transmembrane potential under conditions in which the Ca^{2+}- or Mg^{2+}-ATPase activity is stimulated coincided with the enzymatic hydrolyzing activity as previously described (Caldwell and Haug 1980).

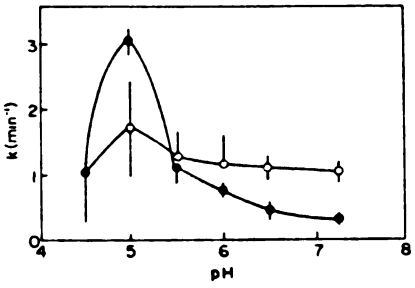

Fig. 2. pH dependence of the rate constant, k, for the development of the transmembrane potential. Vesicles were pre-incubated under the conditions described in the legend to Fig. 1, with 1 m*M* $CaCl_2$ (●), or 1 m*M* $MgCl_2$ (○). Tris-ATP was added to 1 m*M*. Vertical bars show the SD.

Effect of divalent cation concentration on potential development

Development of the membrane potential was more sensitive to changes in $MgCl_2$ concentration than to changes in $CaCl_2$ concentration (Tab. 1). At a physiological pH of 6.5, there was a two-fold decrease in the rate of potential development when the Mg^{2+} concentration was changed from 1 m*M* to 100 μ*M*, with Ca^{2+} absent. Addition of 10 μ*M* Ca^{2+} to either Mg^{2+} concentration did not significantly alter the rate of potential formation. The effect of various Ca^{2+} concentrations in the absence of Mg^{2+} was slight and less than doubled over a three order of magnitude concentration range from 1 μ*M* to 1 m*M*. Where it has been studied, the concentration of free cytosolic Ca^{2+} ranges between 0.1 and 10 μ*M* with Mg^{2+} tending to be 10- to 100-fold more concentrated (Clarkson and Hanson 1980). Mg^{2+} and Ca^{2+} concentrations of 100 μ*M* and 10 μ*M*, respectively, were chosen as representative of physiological concentrations of these divalent cations; the stimulated ATPase activity developed a potential change with a rate constant of 0.08 min^{-1} and is referred to as the control value.

Effect of ionophores, calmodulin, calmodulin antagonists and aluminum on the development of the membrane potential

Results summarized in Tab. 2 indicate that both the H^+/Me^+ ionophore nigericin and the $2H^+/Me^{2+}$ ionophore A-23187 effectively prevented the formation of a transmembrane potential. This is true whether the vesicle suspension is preincubated with the respective ionophore or, as shown in Fig. 3, if the respective ionophore is added after the potential was allowed to develop. In either case the ionophoretic concentration

Tab. 1. Effects of varying divalent cation concentrations on the rate of potential development. Conditions: 0.05 mg vesicle protein ml^{-1}, 0.25 *M* sucrose, 1 m*M* Tris-MES buffer (pH 6.5), 0.3 μ*M* oxonol VI, 16°C. After a stable fluorescence signal was established, Tris-ATP was added to 1 m*M*.

Divalent cation	Rate, k, (min^{-1})±SD
1 m*M* Mg^{2+}	0.116±0.014
1 m*M* Mg^{2+} + 10 μ*M* Ca^{2+}	0.107±0.043
100 μ*M* Mg^{2+}	0.063±0.021
100 μ*M* Mg^{2+} + 10 μ*M* Ca^{2+}	0.080±0.009
1 μ*M* Ca^{2+}	0.019±0.002
10 μ*M* Ca^{2+}	0.029±0.003
100 μ*M* Ca^{2+}	0.038±0.004
1 m*M* Ca^{2+}	0.042±0.003

Tab. 2. Effects of various compounds on the development of the membrane potential. Conditions were as indicated in Tab. 1, except 50 μM Ca^{2+} and 100 μM Mg^{2+} were used. Values are within 5% SE

Compounds	Rate, k, (min^{-1})	% Control activity
No addition (control)	0.08	100
A-23187 (10 μM)	0	0
Nigericin (10 μM)	0	0
Trifluoperazine (10 μM)	0.07	87
Chlorpromazine (10 μM)	0.08	100
$AlCl_3$ (10 μM)	0.08	100
Calmodulin (10 μM)	0.24	300
+ Chlorpromazine (10 μM)	0.09	112
+ Trifluoperazine (10 μM)	0.08	100
+ $AlCl_3$ (10 μM)	0.17	210
+ CCCP (5 μM)	0.04	16

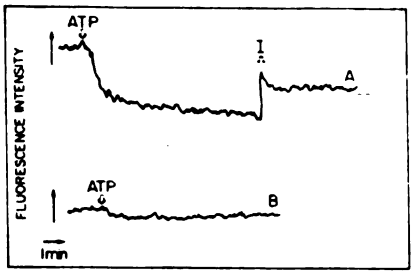

Fig. 3. Effects of ionophores on the development of the transmembrane potential. Vesicles were pre-incubated as described in the legend to Fig. 1, with 100 μM $CaCl_2$. Tris-ATP was added to 1 mM (arrow). (A), addition of 10 μM A-23187 or nigericin (I); (B) pre-incubation with A-23187 or nigericin prior to ATP addition.

was below that which would cause detergent-like disruption of the membranes. The protonophore CCCP is somewhat less effective in preventing potential development than the other ionophores, but it did reduce the calmodulin-stimulated rate by 85%. In the absence of calmodulin, incubation of the vesicle suspensions with the calmodulin-antagonists chlorpromazine or trifluoperazine (Klee et al. 1980) allowed normal development of the ATP-stimulated potential, as did incubation with 10 μM $AlCl_3$. Incorporation of 10 μM calmodulin in the assay suspension increased the rate of potential development by 200% upon ATP activation. Chlorpromazine and trifluoperazine both reversed the calmodulin-dependent activity at a molar ratio of 1:1 for [drug]/[calmodulin], whereas the decrease was only 30% at the same ratio of [aluminum]/[calmodulin].

Aluminum inhibition of the calmodulin-stimulated, ATPase-dependent formation of the transmembrane potential

The viability of seedlings growing in aluminum-toxic soils is known to decrease dramatically as compared with those seedlings growing in soils low in aluminum (Foy et al. 1978). Primary effects of aluminum toxicity apparently occur at the plasma membrane (Hecht-Buchholz and Foy 1981). Thus the Ca^{2+}- and Mg^{2+}-dependent ATPase activity described in this report may serve as a potential marker for detrimental actions of aluminum on membrane maintenance machinery. Figure 4 shows the results of experiments in which vesicle suspensions were incubated with 50 μM Ca^{2+}. Under these conditions the rate of potential build-up did not differ from that rate measured at 10 μM Ca^{2+}. Incubation of the vesicle suspensions with 10 μM calmodulin allowed the potential to form at a rate of 0.24 min^{-1} with 50 μM Ca^{2+} present. Addition of up to 40 μM $AlCl_3$ accelerated the loss of the calmodulin-stimulated activity with a 50% value falling at a molar ratio of 1.4/1 [aluminum]/[calmodulin]. On the other hand, no calmodulin-stimulated activity was detected above 30 μM $AlCl_3$ although these aluminum concentrations did not interfere with the basal ATP-dependent potential development (in the absence of calmodulin).

The observation that the non-calmodulin stimulated activity was not interfered with prompted the investigation of the interaction of aluminum with the isolated protein. The experimental results are reported as the relative fluorescence intensity of ANS, a probe used for monitoring the hydrophobic surface properties of proteins (La Porte et al. 1980). Maximum hydrophobic surface exposure on the protein was achieved at a ratio

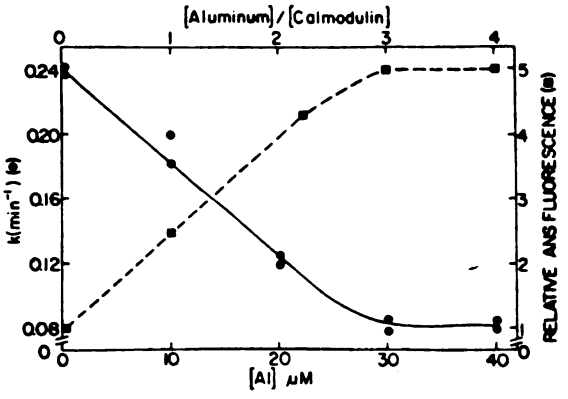

Fig. 4. Effect of aluminum on calmodulin-stimulated potential development or on ANS partitioning onto isolated calmodulin. Vesicles were preincubated as described in the legend to Fig. 1, with 100 μM $MgCl_2$, 50 μM $CaCl_2$, 10 μM calmodulin and various $AlCl_3$ concentrations (●). A rate constant, k, of 0.08 min^{-1} represents non-calmodulin-stimulated activity; a rate constant of 0.24 min^{-1} represents maximal calmodulin-stimulated activity. Relative ANS fluorescence was measured in the presence of 10 μM calmodulin and various $AlCl_3$ concentrations (■). Values are within 5% SE.

of 3:1 [aluminum]/[calmodulin] (mol:mol), the 50% ratio is 1.5/1. Thus, a parallel was established between loss of the calmodulin-stimulated potential development and the loss of structural integrity of calmodulin in the presence of aluminum.

Discussion

The results of this study demonstrate that toxic aluminum ions interfere with calmodulin-stimulated ATPase activity which plays a role in the maintenance of the membrane potential. Calmodulin appears to be the major target for aluminum.

This protein has been shown to mediate calcium regulation in plant enzyme systems (Cormier et al. 1980, Dieter and Marmé 1981). Calmodulin has the capacity to bind four calcium ions at specific sites on each molecule. The resulting conformational changes enhance the hydrophobic surface exposure which is apparently necessary for proper interfacing of calcium-calmodulin and its target protein (Crouch and Klee 1980, Lin 1982). One such target protein seems to be the barley root plasma membrane-bound ATPase found in plasma membrane-enriched vesicles as described in this report, since our results indicate that, at physiological calmodulin concentrations of 10 μM (Klee et al. 1980, Wang and Waisman 1979), electrogenic activity is stimulated by 200% over the activity observed in the absence of calmodulin. As to the calmodulin-stimulated electrogenic activity, our results are consistent with the existence of a membrane-bound ATPase involved in pumping protons or in coupled proton fluxes. The pumping activity leads to the establishment of a membrane potential which, in turn, depends critically on the complete regulatory capacity of calcium-calmodulin. Aluminum-induced changes of calmodulin therefore lead to a reduction in electrogenic activity accompanied by a decreased membrane potential. Aluminum ions interact stoichiometrically with calmodulin (Siegel and Haug 1983). The resulting changes in surface hydrophobicity and helix content lead to a loss of regulatory properties of calmodulin as exemplified by the aluminum-induced inhibition of calmodulin-activated phosphodiesterase activity.

Questions arise whether the ATPase activity present in the vesicle system used in this study is derived solely from the plasma membrane. The activity of the ATPase as measured by inorganic phosphate release is inhibited 50% at a 100 μM N, N'-dicyclohexylcarbodiimide concentration, under conditions of pH and divalent metal concentration similar to those used in our studies (A. Lesniak, personal communication). This result is also consistent with the ATPase activity measured in the plasma membrane of corn leaf, corn root and oat root (Perlin and Spanswick 1981). Possible contamination by other membranes including tonoplast may exist. For this reason we refer to the vesicle system as a plasma membrane-enriched microsome fraction.

For the purpose of the present discussion we refer to the various species of aluminum present in solution collectively as Al. Under the conditions used in the present study we can state that mononuclear, hydrated aluminum species are present, as opposed to polynuclear species existing at higher pH values and elevated aluminum concentrations. We cannot be certain, however, of the charge on these hydrated aluminum species (Baes and Mesmer 1976).

Although ATP represents a potential chelator of aluminum, no inhibitory effect was evident when aluminum was included in the assay system, with calmodulin absent. Viola et al. (1980) showed that yeast hexokinase is strongly inhibited by the presence of Al-ATP with an inhibition constant, K_i, of 0.16 μM, at pH 7. These data supported the earlier findings by Womack and Colowick (1979) who described Al-ATP inhibition of yeast and brain hexokinases. A striking characteristic of the aluminum inhibition in these systems was the observation that when the same amounts of aluminum were added to the reaction mixtures, separately from ATP, more than 10 times as much aluminum was required to get a comparable effect. Since we added ATP separately to the reaction mixture our results therefore cannot be compared to those studies in which ATP pre-mixed with aluminum is added as a substrate. Further support of our results comes from a personal communication by R. Post, cited in Womack and Colowick's article (1979), that aluminum has little effect on the Na^+, K^+-ATPase of guinea pig kidney membranes. We conclude that under our experimental conditions, Al-ATP is not an inhibiting substrate for the ATPase activity.

Our hypothesis is that the Al-calmodulin complex represents a primary biochemical lesion in toxic responses of plants to aluminum. Considering the pivotal role of calmodulin in calcium regulation, aluminum interference is expected to result in severe imbalances of cellular processes, such as maintenance of the membrane potential, cell growth, root elongation and chloroplast function. This hypothesis is consistent with observations that aluminum-induced toxic responses of plants resemble, in part, those occuring as a result of calcium deficiencies.

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Chapter 5

A Thermodynamic and Electron Paramagnetic Resonance Study of Structural Changes in Calmodulin Induced by Aluminum Binding

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A THERMODYNAMIC AND ELECTRON PARAMAGNETIC RESONANCE STUDY OF STRUCTURAL CHANGES IN CALMODULIN INDUCED BY ALUMINUM BINDING

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Bovine brain calmodulin binds 3 mol aluminum per mol protein with dissociation constants in the range of 10^{-7} to 10^{-6} molar. EPR spectra of spin-labelled calmodulin provide data indicating that aluminum binding causes decreased probe immobilization as compared to the effects of calcium binding. This result of aluminum binding indicates that Al-calmodulin is a more random, open polypeptide relative to the structure of Ca^{2+}-calmodulin. Calorimetric measurements of aluminum binding provide data showing that the first mol of aluminum bound is accompanied by the largest enthalpic change (-3.9 kcal mol^{-1}), whereas binding of the second and third mol of aluminum are each entropically driven.

Calmodulin is an important Ca^{2+}-dependent regulating protein in almost all eukaryotic tissues and organs (1-4). Recently it was suggested that aluminum ions, which are toxic to plants and animals in low concentration, may exert their toxic properties by interacting with calmodulin (5). This protein loses its structural integrity upon the stoichiometric binding of aluminum and ceases to retain the capacity to regulate Ca^{2+}-calmodulin dependent phosphodiesterase (5) or a Ca^{2+}-calmodulin dependent ATPase in the barley root plasma membrane (6).

To further investigate the changes in calmodulin induced by aluminum binding we have analyzed the thermodynamic properties of this process using calorimetric methods and equilibrium dialysis. Correlation times for covalently attached spin probes on the protein were calculated from EPR spectra to assess relative changes in protein structure in response to metal binding. Data are presented showing that three mol of aluminum bind specifically to each mol of calmodulin; binding of the first mol of aluminum bound is enthalpically driven in contrast to the second and third mol

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bound which are entropically driven. When bound by aluminum the protein apparently takes on an open, more random conformation as compared to the effects of calcium binding.

MATERIALS AND METHODS

Sources: Bovine brain acetone powder, Tris, from Sigma Chemical Co. (St. Louis, MO); Affigel-Phenothiazine from Bio-Rad Laboratories (Richmond, CA); $AlCl_3$, $CaCl_2$ from Mallinckrodt Science Products (St. Louis, MO). 3-[α-iodoacetamido]-2,2,5,5-tetramethyl-1-pyrrolidinyl oxyl from Syva Corp. (Palo Alto, CA). All other chemicals were of the highest purity that were commercially available.

Methods: Calmodulin was isolated from bovine brain acetone powder and prepared to be metal-free as previously described (5). Protein concentrations were determined spectrophotometrically (8). Spin labelling of calmodulin was accomplished using the method of Hewgley and Puett (9). Microcalorimetric data were collected using a LKB 210 batch microcalorimeter equipped with a pair of gold mixing cells and were corrected for heats of mixing as described (11). The experimental temperature was maintained at 23.85 ± .01°C. Equilibrium dialysis experiments were conducted and aluminum analyzed as previously described (5). Analysis of the EPR signal from a Varian X-band EPR spectrometer E-112 using a Varian 620/L-100 computer showed that 1.34 spin labels were bound per protein molecule. Correlation times (τ_c) were calculated from EPR spectra using the following relationship (10):

$$\tau_c = 6.5 \times 10^{-10} w_o [(h_o/h_{-1})^{1/2}-1] \text{ sec}$$

where w_o is the midline width (gauss), and h_o and h_{-1} are the peak heights of the mid- and high-field lines, respectively. Limitations of this equation are noted as described by Melhorn et al (10) and the calculated values are applied as standard quantitative measures for comparison of spectra.

RESULTS AND DISCUSSION

As shown in Fig. 1, the calculated values of τ_c for Ca^{2+} addition to calmodulin increased 6% beyond the value for the metal-free protein and saturated between a ratio of 4 and 5 mol calcium per mol calmodulin. This change in τ_c indicates increased immobilization of the spin label and is consistent with compaction of the protein upon binding of calcium (1-4). In contrast, as a function of increasing τ_c calculated for increasing amounts of aluminum decreased the immobilization of the spin label indicating increased randomness of the polypeptide region near the spin probe. These results are consistent with other aluminum-induced structural changes in calmodulin (5) including decreased helical content, increased random coiling and an increased hydrophobic surface expression. Calcium binding has been shown to contrast these changes; the helical content increases, random coiling decreases and there is a small increase of hydrophobic surface area (1-4).

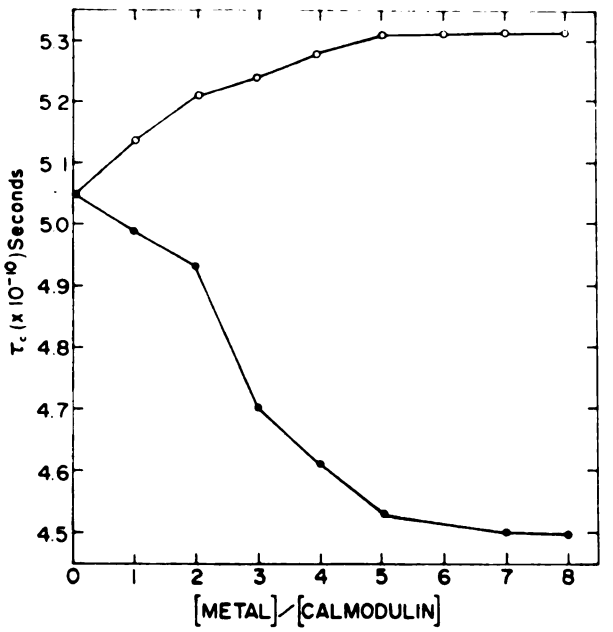

Fig. 1. Changes in spin-probe mobility of covalently labelled calmodulin due to metal addition. Values for τ_c (correlation times) were calculated as described in MATERIALS AND METHODS. Changes due to aluminum (●) or calcium (O) addition to 10 μM calmodulin at pH 6.5 are shown. Values are shown for at least two separate trials.

For the purpose of the present discussion we refer to the various species of aluminum present in solution collectively as aluminum. Under the conditions used in the present study we can state that mononuclear, hydrated aluminum species are present, as opposed to polynuclear species existing at higher pH values and elevated aluminum concentrations. We cannot be certain, however, of the charge on these hydrated aluminum species (7).

Thermodynamic functions associated with aluminum binding to calmodulin are summarized in Table 1. Binding constants were calculated from the equilibrium dialysis data presented in Fig. 2. The enthalpic contribution for the first mol of aluminum bound is -3.9 kcal/mol, both opposite in sign and greater in magnitude than that for the next two mol aluminum bound. The calculated entropic contribution increased during the binding of the second and third mol aluminum bound; the total entropic contribution is 103.8 e.u.; only 21.9 e.u. are contributed by the binding of the first aluminum mol bound. These results differ from the effect of Ca^{2+} binding to

Table 1. Thermodynamic parameters associated with the binding of aluminum to bovine brain calmodulin*

Aluminum Binding Site(i)	K_{Al_i}**	ΔG_i	ΔG_i^o	ΔH_i^o	ΔS_i	ΔS_i^o
		Kcal mol^{-1}			cal deg^{-1} mol^{-1}	
1	1.2×10^{-6}	-9.0	-10.4	-3.9	13.9	21.9
2	9.9×10^{-7}	-8.2	-10.5	+1.2	31.4	39.4
3	1.3×10^{-7}	-9.4	-11.8	+0.9	34.5	42.5

* ΔG_i was calculated from the relationship: $\Delta G_i = -RT\ln K_{Al_i} = \Delta H_i^o - T\Delta S_i$ where K_{Al_i} is expressed in terms of molar concentrations. ΔS_i^o, the unitary changes in entropy, were calculated from: $\Delta S_i^o = \Delta S_i + 7.98$ (11, 16). The unitary free energy change, ΔG_i^o, was then calculated as $\Delta G_i^o = \Delta H_i^o - T\Delta S_i^o$ for T = 298°K at pH 6.5.

** K_{Al_i} were calculated from equilibrium dialysis data.

Troponin C, a calcium binding protein similar in nature to calmodulin (11). In that study of Troponin C, calcium binding to the four available sites on the protein have enthalpic contributions of -7.7 kcal mol^{-1} for each site and the entropic contribution

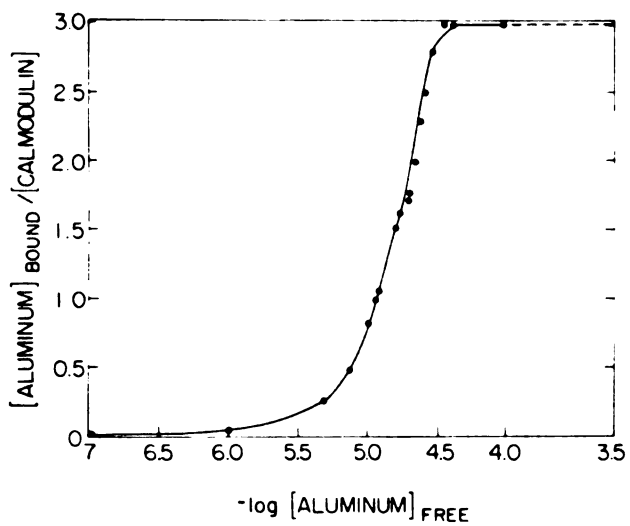

Fig. 2. Binding of aluminum to calmodulin. One-ml sample volumes of 10 μM calmodulin were dialyzed against various concentrations of aluminum chloride at pH 6.5. Aliquots were analyzed (5) for aluminum content both inside and outside of the dialysis bag after 24 h.

decreases from 14.7 e.u. for the first two sites to 8.0 e.u. for the third and fourth sites. In the case of Troponin C, Ca^{2+} binding is in part an enthalpically driven process.

The results of our experiments complement the spectroscopic work which detailed structural alterations of calmodulin induced by aluminum binding (5). Explanation of the observed thermodynamic changes during aluminum binding to the protein comes from the unique hydration properties of this metal. Both aluminum and calcium have a primary coordination number of six representing the effective hydration number of the innermost hydration shell (12). It has been discussed that only a single water molecule exists at the high affinity Ca^{2+}-binding site of Troponin C (13) or the Ca^{2+}-binding sites of calmodulin (14). Therefore, the binding of calcium by these proteins is accompanied by the release of five coordinated water molecules and an entropy change of about 15 e.u. (11). The strength of the ion/water coordination bonds is three-fold higher for aluminum than calcium; i.e., 1144 kcal/mol Al^{3+} vs. 399 kcal/mol Ca^{2+} (15). Also, the value of the formal charge over ionic radius and the intermediate electronegativity are both a factor of 2 higher for aluminum relative to calcium (14), and the number of water molecules in the outer hydration shells of aluminum is significantly higher than for calcium (12). Together, these physical data support the conclusion that aluminum ions differ appreciably from calcium ions in their interaction with calmodulin. Under our experimental conditions, mononuclear, hydrated aluminum species interact with calmodulin and promote an open, solvent-rich, disordered polypeptide region, effects which contrast those of calcium binding to this protein.

The observed enthalpy change for the first mol aluminum bound is composed of two terms, ΔH for hydrogen bond breakage and ΔH for solvation. Since the ΔH for hydrogen bond breakage is positive, the measured enthalpy change must have as a major contribution an enthalpic change associated with increased solvation of the protein upon binding the first mol of aluminum. As discussed above, interaction of calmodulin with the highly hydrated aluminum ions accounts for the observed enthalpy change.

The results of this study indicate that the aluminum calmodulin interaction seems to result from the unique hydration properties of this metal. This type of interaction may serve to explain the potent, biologically toxic properties of aluminum.

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Chapter 6

Summary

The results presented in chapters 2 and 3 show that aluminum ions interact specifically with the calmodulin molecule. Three mol of aluminum are bound per mol of calmodulin. Thermodynamic data regarding this binding as presented in chapter 5 indicate that the binding is tight with K_d's in the range of 0.1 to 1 μM. Calorimetry indicates that entropic changes accompany aluminum binding, a result that is complementary to spectroscopic data showing the expression of a large hydrophobic surface and a helix-coil transition within the protein that also accompany aluminum binding. In the most fundamental sense, calmodulin is denatured upon binding aluminum, even with calcium present.

The reason that calmodulin loses its structural integrity through binding aluminum rests in the unique physicochemical characteristics of the highly solvated aluminum ion. Both aluminum and calcium have similar binding affinities for calmodulin and both have an inner hydration shell composed of six water molecules (Hindman and Sullivan, 1971). Five of these water molecules coordinated to calcium are released upon its binding to Troponin C, a calcium binding protein that shares sequence homology in the calcium binding loops with calmodulin (Wang et al, 1981). The strength of these coordinated bonds is appreciably higher for aluminum than for calcium; 1144 Kcal/mole as compared with 399 Kcal/mole, respectively (Matheja and Degens, 1971). Additionally, the ratio of the formal charge to ionic radius and the intermediate electronegativity are both a factor of

two higher for aluminum than for calcium (Noller, 1982). These physical data lead to the expectation that aluminum should interact differently with calmodulin than should calcium, and this conclusion is supported by data presented in chapters 2,3 and 5 showing that aluminum promotes dissintegration of the stabilizing forces involved with maintaining the structural integrity of the protein.

The specific amino acid residues to which aluminum becomes bound are presently not known. As far as calcium is concerned, four specific binding loops exist in the protein and this metal appears to be coordinated to oxygen ligands with its mobility becoming restricted when bound (Andersson et al, 1982, Krebs, 1981). Aluminum also forms stable complexes wtih electronegative ions such as oxygen and nitrogen. In contrast with calcium, the higher electronegativity of aluminum would result in an increased covalent character of the coordinated bond established, a conclusion which is borne out by the data indicating that the binding affinity for calnodulin is 5 to 10 fold higher for aluminum than for calcium as presented in chapters 2, 3, and 5.

The effects exerted by aluminum on calmodulin must extend to the intramolecular bonds that maintain this protein's structure in order to cause the observed physical alterations. Similarly, the interaction of calcium with calmodulin must affect intramolecular bonds with effects opposite to those caused by the binding of aluminum. It is thought that upon binding calcium, negative charges on the

protein are neutralized. In turn, weakened constraining forces result permitting the formation of additional helical elements. Ultimately the calcium-calmodulin complex becomes a more compact structure than the metal-free calmodulin (Seamon, 1980). The interaction of aluminum is more complex particularly because of the highly hydrated character of this ion as previously discussed. The results presented in chapters 2 and 3 show that aluminum binding leads to a helix-coil transition that is accompanied by a strong enhancement of the protein's hydrophobic surface domains. Simply stated, the hydrated aluminum ions promote open, solvent-rich, disordered polypeptide regions contrasting the effects of calcium.

The biological consequences of aluminum-induced alterations in calmodulin's structure are described in chapters 3 and 4. Calcium-calmodulin stimulation was lost in the presence of aluminum for phosphodiesterase activity and for the electrogenic ATPase activity in barley root plasma membrane vesicles. As a result of the loss in calmodulin's flexibility when aluminum is bound, its ability to correctly interface with and regulate various target proteins are impaired. Thus, aluminum-calmodulin complex represents a primary biochemical lesion in aluminum toxicity. Considering the pivotal role of calmodulin in calcium regulation, aluminum interference is expected to result in severe imbalances of cellular processes of plant cells such as maintenace of the membrane potential, cell growth, root

elongation and chloroplast function. This is consistent with observations that aluminum-induced toxic responses of plants resemble, in part, those occuring as a result of calcium deficiency.

The data presented in this dissertation represent the first reports of a specific biochemical target for aluminum under conditions of physiological relevence. This is not to say that aluminum interaction with calmodulin is the only toxic interaction that involves aluminum. However, this study does serve to elucidate a model on which other toxic aluminum interactions within the living organism may be based. The recent report that metal toxicity might be correlated with calmodulin inhibition (Cox and Harrison Jr., 1983) adds support to this model.

The path leading to the discovery that aluminum attaches strongly to calmodulin and thus may exert toxic effects in the cell was less than straightforward. At first, inhibition of the barley root plasma membrane ATPase via complexation of ATP by aluminum was investigated. There was a precedent for this in that yeast hexokinase is inhibited by Al-ATP (Viola et al, 1980). Under the conditions in which the ATPase activity of the isolated vesicles was assayed (Chapter 4), aluminum interfered with the colorimetric assay for released phosphate which necessitated a better way for monitoring the enzymatic activity. The approach used assumed that the ATPase activity was electrogenic, that is, the ATPase acts as an ionic pump at the plasma membrane.

Fluorescent, potential-sensitive probes were chosen to assay this system of sealed vesicles because these vesicles were shown in preliminary studies not to be influenced by aluminum or other metal addition. Optimization of the system as reported in chapter 4 involved manipulation of the divalent metal concentrations and included calmodulin, previously shown to enhance the enzymatic activity of the ATPase (Caldwell and Haug, 1980).

Addition of aluminum to the optimized system consistently lowered the electrogenic activity, but not below a basal value established in the absence of calmodulin (Chapter 4). This was the first indication that the interaction of aluminum involved calmodulin. Introductory studies of the effect of aluminum on isolated calmodulion indicated that the protein's hydrophobic surface expression was greater when aluminum interacted with it than when calcium interacted with it. Only as more data were collected indicating that calmodulin became denatured upon the specific binding of aluminum did it become clear that the interaction may represent a primary biochemical lesion during aluminum intoxication. Once the specificity and potency of aluminum interaction with calmodulin became established, it was logical to assume that because calmodulin is ubiquitous in eukaryotic cells, and because plants and animals including man suffer adverse reaction to aluminum exposure, the common link was calmodulin.

It is known in some plants that aluminum tolerance is genetically linked (Foy et al, 1978). Because the aluminum-calmodulin complex represents a primary lesion in the toxic responses of plants to this metal, future research must attempt to discover what protective mechanisms are available to those genetically resistant plant varieties. These defenses might include enhanced levels of aluminum-specific chelators that can sequester aluminum away from any interaction with calmodulin (Suhayda and Haug, 1983), amino acid substitutions in calmodulin that maintain structural stability after aluminum is bound or extracellular compounds that prevent aluminum entry into the cell. When the protective processes that prevent aluminum-calmodulin interaction are elucidated, therapeutic methods of relieving this stress should not be far behind. It is hoped that this study is a first step toward that end.

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APPENDIX 1

STRUCTURAL DISTINCTIONS BETWEEN BARLEY AND BRAIN CALMODULIN: A STUDY OF STRUCTURAL CHANGES ACCOMPANING CALCIUM AND ALUMINUM INTERACTION WITH BARLEY CALMODULIN

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Calmodulin isolated from barley, like that from other plant sources contains one tyrosine and one cysteine residue whereas calmodulin isolated from animal sources contains two tyrosine residues and does not contain cysteine. Other differences in amino acid content are reflected in decreased α-helical content of barley as compared to bovine brain calmodulin. Application of calcium increases the protein's helical content and aluminum application decreases the helical content, each to a similar extent regardless of protein source. Hydrophobic surface exposure is also enhanced upon metal interaction with calmodulin when monitored with 8-anilino-1-naphthalene sulfonic acid. These results indicate that aluminum-calmodulin represents a primary biochemical lesion of aluminum intoxication in both plants and animals.

INTRODUCTION

Aluminum interaction with calmodulin has recently been focused upon as a primary biochemical lesion that occurs during the toxic response of eukaryotic cells to this metal (1-3). This research has used bovine brain calmodulin as a model system due to the conserved sequence of this ubiquitous calcium-dependent regulation protein (4). Differences exist, however, in the amino acid composition between plant calmodulin and animal calmodulin, most notably in the occurence of a cysteine residue in the plant protein that is

not found in the animal protein (5). Other differences include a higher amount of proline in the plant protein and one instead of two tyrosine residues as found in animal calmodulin. Because calmodulin is known to undergo structural alterations upon binding metals including aluminum and calcium (1,2,6), it is important to understand how compositional differences between plant and animal calmodulin affect the structural response of each to metal binding.

Changes in the barley protein's circulr dichroism spectrum in the ultraviolent wavelength region upon metal binding show that calcium induces a coil-helix transition; these results are qualitatively identical with results of a similar study utilizing bovine brain calmodulin (1). More hydrophobic surface exposure occurs on the barley protein when aluminum application is compared with calcium application; these results are also qualitatively similar to results using bovine brain calmodulin. Electrophoretic analysis using denaturing polyacrylimide gel electrophoresis (PAGE) shows that the molecular weight of barley calmodulin is virtually identical to bovine brain calmodulin whereas analysis using non-denaturing PAGE and protein bound with calcium or aluminum shows that the electrophoretic mobility is dependent on the type of metal bound.

MATERIALS

Sources: Tris, from Sigma Chemical Co. (St Louis, MO), Affigel-Phenothiazine and SDS-PAGE Low Molecular Weight

Standards from Bio-Rad Laboratories (Richmond, CA), $AlCl_3$ and $CaCl_2$ from Mallinckrodt Science Products (St. Louis, MO), sodium salt of ANS from K&K Laboratories (Plainview, N.Y.). All other chemicals were of the highest quality available.

Methods: An acetone powder from barley (Hordeum Vulgare var. Conquest) was prepared by excising shoots of plants grown at pH 6.5 in 250 uM $CaSO_4$ possessing intact coleoptiles, freeze-drying these and washing the resulting powder with acetone to remove soluble pigments. The acetone powder was stored at 0°C. The procedure for isolating calmodulin from this material was the same as that described for bovine brain acetone powder (1). 33 g of starting material yielded 14 mg of calmodulin which ran as a single band using SDS-PAGE and the silver stain technique (7). Circular dichroism measurements were made using a Jasco spectropolarimeter, model ORD/UV/CD-5 as previously described (1). Fluorescence measurements were made as previously described using a SLM 4000 spectrofluorimeter; each point was signal-averaged over 10 scans, 10 times to provide data points falling within 5% standard error of the mean value. Amino acid analysis was performed using a modified Dionex Amino Acid Analyzer with a DC5A microcolumn. Electrophoresis was done on 15% acrylimide gels; denaturing gels included 0.4% SDS.

Abbreviations: ANS, 8-anilino-1-naphthalene sulfonic acid; SDS, sodium dodecyl sulfate; CD, circular dichroism; Tris, Tris-(hydroxymethyl)-aminomethane.

RESULTS AND DISCUSSION

As shown in Fig. 1, the electrophoretic mobility of bovine brain caomodulin and barley calmodulin under denaturing conditions shows calculation that the molecular weights for each lie between 16,500 and 17,300 agreeing with published reports (4,5). Electrophoresis under non-denaturing conditions as shown in Fig. 2 indicate that structural changes induced by metal binding with calmodulin result in differing mobilities dependent on whether the protein is metal free, bound with calcium, or bound with aluminum. Barley calmodulin is less electrophoretically mobile than brain calmodulin under these conditions regardless of pretreatment indicating that structural differences exist between the two molecules; however, charge differences under these conditions become a major factor and may be responsible to some extent for the different mobilities of the metal-protein complexes.

Metal-free bovine braine calmodulin has a helical content of 37% (1) calculated using $[\theta]_{222}$ from CD spectra and applying the method of Chen et al. (8). Under identical conditions, metal-free barley calmodulin has a helical content of 26% (Tab. 1, Fig. 3). Application of calcium caused increased helical formation for both barley and brain calmodulin with net helix content increases of 12% and 18%, respectively at saturation. Aluminum application caused helical loss for both barley and brain calmodulin with net helix losses of 42% and 40%, respectively. The physical

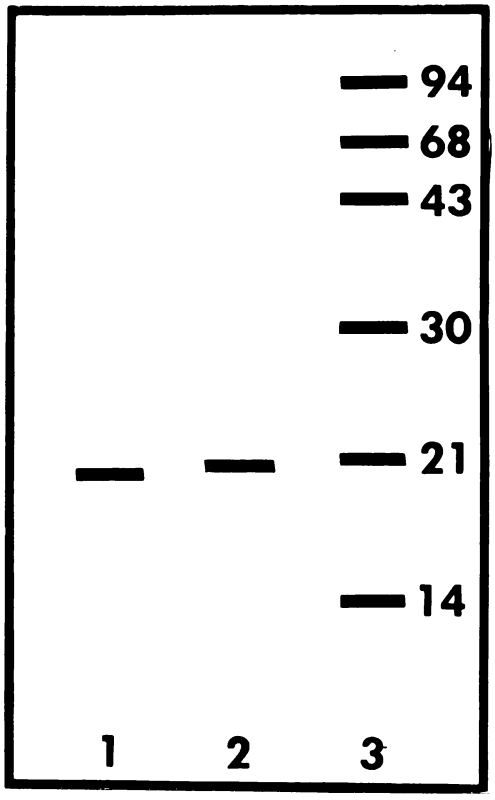

Fig. 1. Electrophoresis of calmodulin on 15% acrylimide gels in the presence of SDS. 10 ng of barley calmodulin (lane 1) and bovine brain calmodulin (lane 2) were run with SDS-PAGE molecular weight standards (lane 3) Numbers next to lane 3 indicate standard molecular weights. Proteins were visualized using the silver stain technique (7).

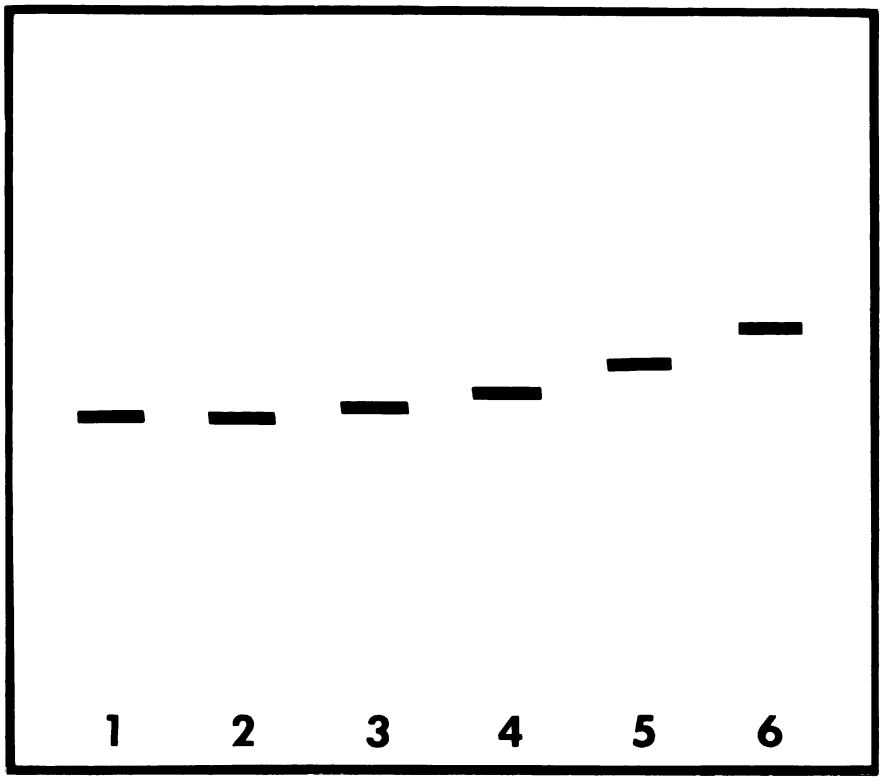

Fig. 2. Electrophoresis of calmodulin on 15% acrylimide gels under non-denaturing conditions. 10 μM stock solutions of calmodulin from bovine brain or barley were prepared in metal free buffer or in buffer containing 100 μM $AlCl_3$ or $CaCl_2$. 10 ng of each type of treated calmodulin were electrophoresed. Bovine brain calmodulin in metal free, calcium or aluminum treated buffers are in lanes 1,3, and 5, respectively. Barley calmodulin in metal free, calcium or aluminum treated buffers are in lanes 2,4 and 6, respectively. Proteins were visualized as described in Fig. 1.

Table 1. Changes in $[\theta]_{222}$ and helical content of calmodulin derived from bovine brain and barley induced by metal binding

	$[\theta]_{222}$ (mdeg cm /dmole)		% α-Helix	
	Barley	Brain	Barley	Brain[b]
-Metal	-10,182	-13,571	26	37
Ca_{sat}	-11,283	-16,100	29 (+12)	44 (+18)
Al_{sat}	- 7,086	-10,280	15 (-42)	22 (-40)

[a] numbers in parentheses indicate percent change from initial helical percent

[b] data from Siegel and Haug (1983a)

Results were calculated by the method of Chen et al. (8)

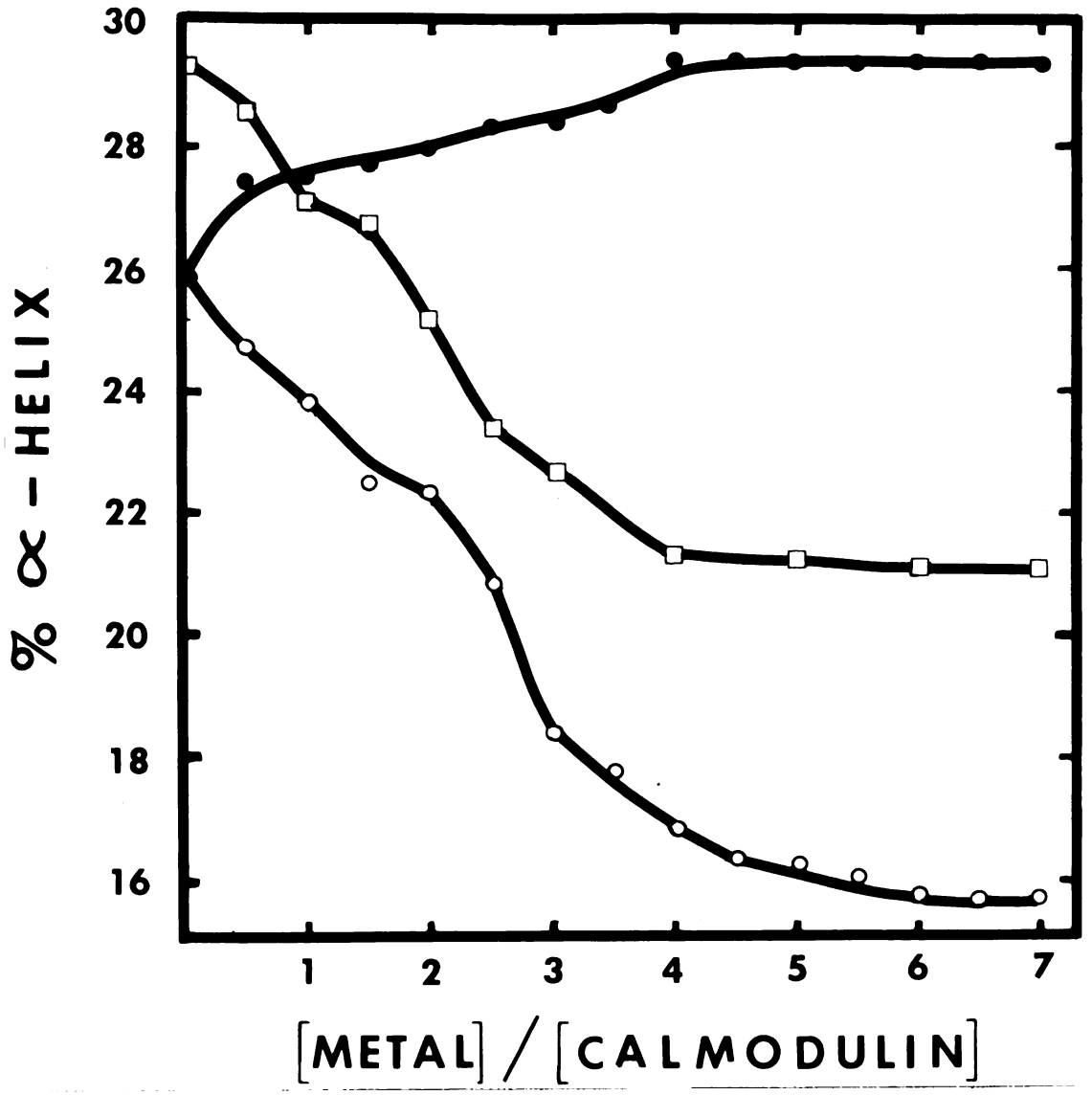

Fig. 3. Changes in the helical content of 10 μM barley calmodulin titrated with $CaCl_2$ (●), $AlCl_3$ (○) or $AlCl_3$ in the presence of 70 μM $CaCl_2$ (□). All procedures were done in 10 mM Tris, pH 6.5.

reasons for these structural changes have been previously discussed for aluminum interaction with bovine brain calmodulin (1), and the statement that highly hydrated, aluminum ions promote open, solvent-rich, random polypeptide regions in barley calmodulin applies.

The structural changes induced by aluminum binding to bovine brain calmodulin was the same in the absence of calcium or in the presence of saturating amounts of calcium (1). Barley calmodulin, however, had more helix remaining if calcium was present at saturating amounts that when it was absent (Fig. 3). There was a net helical loss of 30% when aluminum was applied with calcium present; this effect saturated at a value of 21.3% as compared with a saturating value without calcium present of 15%. Differences in the amino acid composition between the two types of calmodulin are probably involved and are reflected in increased structural stabilization of the barley protein as compared to the animal protein when bound with calcium.

Hydrophobic surfaces on the calmodulin molecule are expressed in the presence of calcium and are though to provide the interface for regulating target protein activity (9). Aluminum application greatly enhanced their exposure in bovine brain calmodulin (1,2) and the aluminum induced change was insensitive to the presence of calcium. Fig. 4 shows that aluminum application increased the hydrophobic surface exposure relative to that of calcium application; exposure of this surface by aluminum was approximately

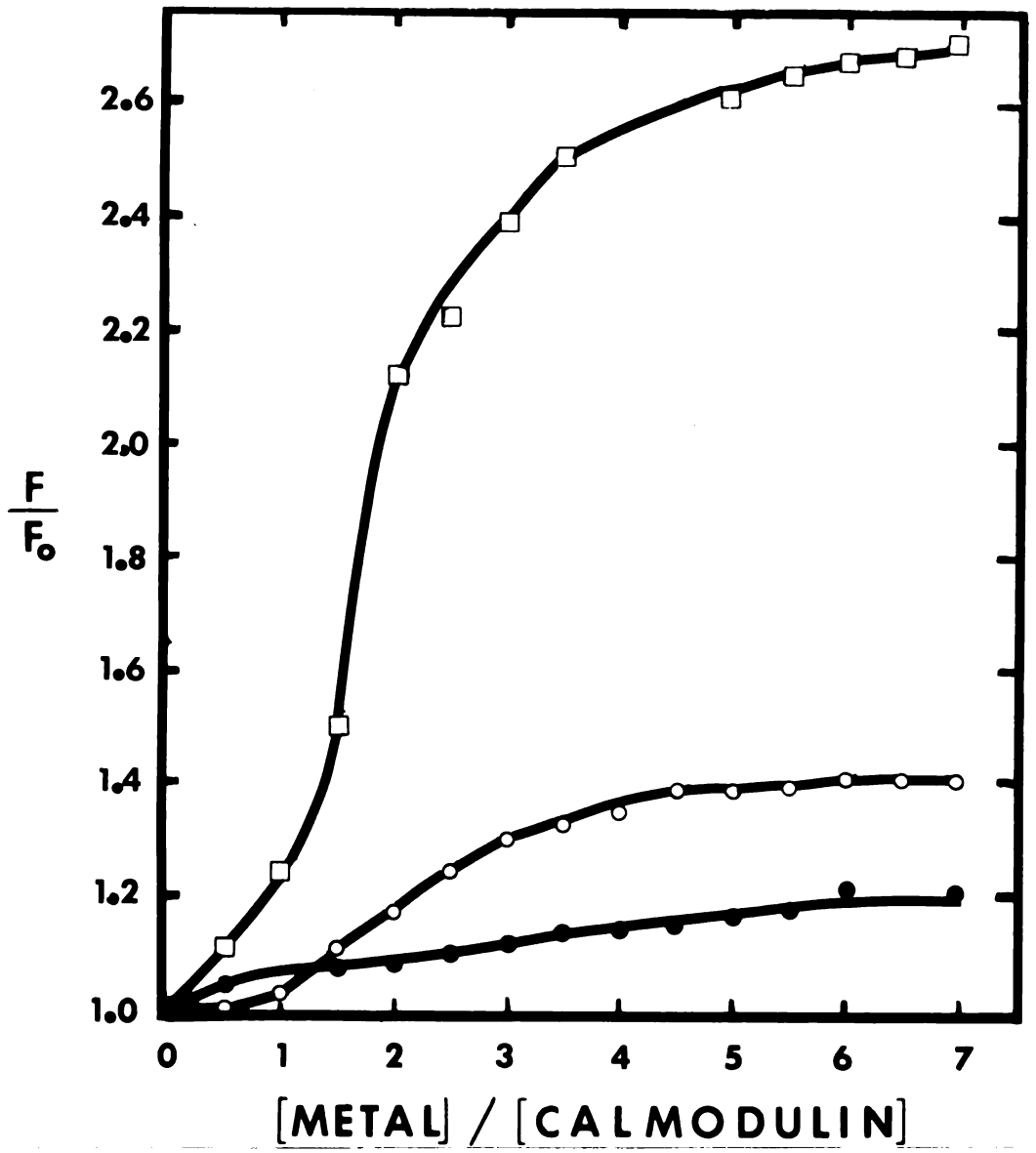

Fig. 4. Changes in ANS fluorescence of 10 μM barley calmodulin titrated with $CaCl_2$ (●), $AlCl_3$ (○), or $AlCl_3$ in the presence of 70 μM $CaCl_2$ (□). Ordinate values are treatment fluorescence value, F, divided by the initial fluorescence value for the individual treatment, F_o. All procedures were done in 10 mM Pipes buffer, pH 6.5, 2 μM ANS. Excitation and Emission monochrometers were set at 360 ± 4nm and 490 ± 4nm, respectively.

double in the presence of calcium than in the absence of calcium. Similarly to the CD data, structural differences reflecting amino acid differences between the animal and plant proteins are probably involved.

Amino acid composition of barley and brain calmodulin are presented in Table 2. In light of the decreased helical content observed for barley calmodulin as compared to brain calmodulin, it is important to note that cysteine and proline whcih destabilize the helical structure of proteins are present in higher amounts in the plant protein than in the animal protein. Cysteine has been shown to occur only in plant calmodulin (5). Thus, the observation that the plant protein has less helical content than the animal protein is due to the presence of these helix destabilizing amino acid residues.

The results of this study indicate that the interaction of aluminum with calmodulin is as potent whether the protein source is plant or animal. Barley calmodulin which possesses more helix-destabilizing amino acid residues than bovine brain calmodulin has less α-helical content than bovine brain calmodulin, but undergoes equivalent structural changes in the presence of calcium or aluminum. Structural stabilization of the calcium-calmodulin complex from barley as compared with bovine brain may result from the different amino acid composition. An aluminum-calmodulin complex has been hypothesized to be a key lesion in the toxic responses of plants to this metal. It is known that some species of

Table 2. Amino acid content of calmodulin

Amino Acid	Mole Percent barley	Mole Percent bovine brain[a]
ASP	16.0	15.5
THR	6.7	8.1
SER	5.5	2.7
GLU	16.1	18.2
GLY	9.1	7.4
ALA	7.0	7.4
VAL	7.5	4.7
MET	2.4	6.1
ILU	5.0	5.4
LEU	8.0	6.1
TYR	0.6	0.6
PHE	4.8	5.4
HIS	0.8	0.6
LYS	4.8	4.7
ARG	2.0	4.0
PRO	2.3	1.3
CYS	0.6[b]	0.6

[a]from Cormier et al (1980)
[b]not directly determined

barley posses a genetic tolerance to aluminum intoxication (10). Other amino acid substitutions in the calmodulin molecule may further stabilize the protein against aluminum induced structural changes and thus may be the basis for aluminum tolerance.

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