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ORGANIC ACIDS PREVENT ALUMINUM-INDUCED CONFORMATIONAL CHANGES IN CALMODULIN

Charles G. Suhayda[1,2] and Alfred Haug[2]

[1]Department of Botany and Plant Pathology and [2]Pesticide Research Center, Michigan State University, East Lansing, MI 48824

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

RESULTS

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

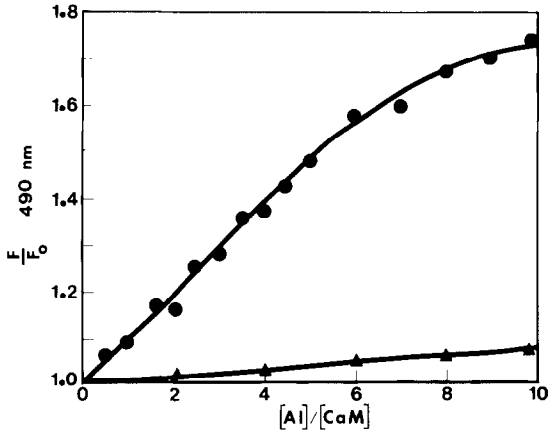

Figure 1. Aluminum-induced hydrophobic surface exposure of bovine brain calmodulin, in the absence (●) and in the presence of 100 μM citrate (▲). The concentration of calmodulin was 10 μM in 10 mM PIPES buffer, pH 6.5. The fluorescent, hydrophobic probe 8-anilino-1-naphthalene sulfonate (ANS) was added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

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

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

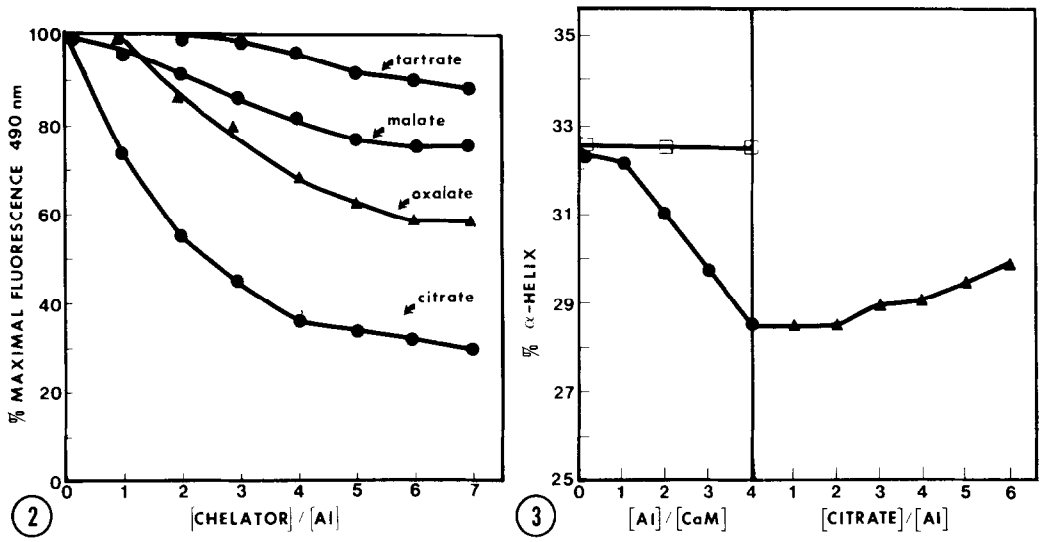

Figure 2. Decrease of aluminum-induced hydrophobic surface exposure of calmodulin, measured as maximal fluorescence intensity of 8-anilino-1-naphthalene sulfonate (ANS), following titration with organic acids as aluminum chelators. The concentration of calmodulin was 10 μM in 10 mM PIPES buffer, pH 6.5.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

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

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

DISCUSSION

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

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

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

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