This is to certify that the

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Biophysical Aspects of Aluminum Intoxication

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Christopher Pat Weis

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BIOPHYSICAL ASPECTS OF ALUMINUM INTOXICATION

by

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ABSTRACT

BIOPHYSICAL ASPECTS OF ALUMINUM INTOXICATION

By

Christopher P. Weis

Continuing acidification of aqueous and terrestrial environments has caused mobilization of large quantities of toxic aluminum ions. This highly reactive metal makes up 8% of the Earth's crust but, as yet, no known biological function has been defined for the ion. Slow accumulation of aluminum has been suggested to cause neurological, bone and endocrinological disorders in humans. Realization of full agricultural potential in aluminum stressed soils, which make up nearly two thirds of available arable land, will require understanding and elimination of aluminum toxicity. This thesis is directed toward defining mechanisms of aluminum toxicity from a biophysical perspective. Studies included focus on the ability of toxic aluminum species and altered pH to damage or modulate association between the calcium regulatory protein calmodulin and model target proteins. The role of pH and aluminum in alteration of plasma membrane lipid order and fluidity is investigated

using time resolved fluorescence polarization. In chapter two, using circular dichroism, time resolved fluorescence, and enzyme assay, pH is shown to modulate the conformation and function of calmodulin within the physiological pH range. Chapter three and appendix B define the role of aluminum in disturbing the binding between calmodulin and melittin or mastoparan, model targets for calmodulin function. Membrane disturbances induced by aluminum and altered pH are investigated in chapter four and appendix C. Time resolved dynamic motion of the membrane probe DPH, and proton sensitive fluorescence probes are used to monitor proton pumping and membrane fluidity in response to aluminum and acid stress.

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CHAPTER I

LITERATURE REVIEW AND SUMMARY OF RESEARCH

CHRISTOPHER WEIS

Introduction:

At this writing, acidification of soil and aqueous environments of northern America and Eurasia continues at an alarming rate. This process of acidification strongly favors the entry of large quantities of toxic aluminum species into the biosphere [1,2,3,4]. Freed into a mobile form, aluminum enters these environments where it presents a formidible barrier to growth and reproduction of aquatic organisms as well as preventing successful use of almost two thirds of the Earth's arable soils. For example, teleosts suffer extremes of edema and hyperplasia of gill tissue ultimately resulting in death when exposed to micromolar concentrations of aluminum. Under acidified conditions, aluminum toxicity has been reported to be the primary cause of death in these animals [5,6,7]. Crop production is impeded under acidified conditions due to increased proton concentration as well as the presence of toxic concentrations of aluminum (see appendix C). The pathogenic effects of aluminum with regard to human health is of continuing and as yet unresolved concern.

Until recently, the significance of aluminum toxicity in humans has been largely overlooked. Continual, unavoidable exposure to aluminum, the lack of profound acute toxicity of the metal and slow accumulation of aluminum all have contributed to this indifference. Attention was focused on

the potential problem of aluminum toxicity when it was determined in the late 1970's that several dialysis patients were suffering severe consequences from dialysis water which had high levels of aluminum. Since that time, accumulation of aluminum in human nervous and bone tissue has been causally related to dialysis encephalopathies and dialysis osteomalacia [8,9,10]. Accumulation of aluminum has been related to formation of heterochromatin and has been proposed as an important cause of cellular aging and death [11]. Diseases with proposed connections to aluminum accumulation include Senile Dementia of the Alzheimer's Type [12,13,], Amyotrophic Lateral Sclerosis [14], and Idiopathic Parkisonism [11]. In light of the rapidly increasing environmental acidification and human health threat a better understanbding of the mechanisms by which aluminum wreaks morbidity and mortality on living systems is essential.

It is the purpose of this dissertation to explore the biophysical aspects of aluminum intoxication and its relation to acidification in an effort to aid in an understanding of the interaction between toxic aluminum ions and living systems. Emphasis is placed on investigation of motional characteristics of cellular components and the usefulness of motional alterations for perceiving and predicting toxicity.

Physico-chemical aspects of aluminum:

In nature, aluminum is found only in the Al(III) oxidation state. Ligand binding characteristics of the ion are central to an understanding of the interaction of aluminum with organic components of living cells (see appendix A). Key factors influencing complex formation include the high charge to radius ratio (58.8), the resulting strong polarizability of the ion and its coordination geometry. The free aluminum ion at highly acidic pH is trivalent and hexahydrated forming an octagonal configuration with water molecules. Neutralization of the solution will promote the formation of hydroxo complexes as aluminum hydrolyzes the surrounding water molecules. The result is the formation of polynuclear aggregates of aluminum which increase in size and complexity until a white precipitate is formed. Maturation of such complexes at neutral pH can take years. Acidification results in a reversal of complex formation and increased concentrations of free, toxic, single, double and triple charged monomers. [15]. Aluminum ions, like calcium ions, prefer oxygen containing ligands [16,17]. Consequently, acidic oxygens of membrane lipids, carboxyl groups of proteins and chromatin material make ideal target ligands for cationic aluminum.

Absorbtion and Distribution:

A majority of available information concerning the absorption and distribution of aluminum in living systems has been collected in response to the human health threat that this ion poses. Total body content for healthy individuals is reported to be between 30 to 50 mg [18,19]. One half of this amount is expected to be found in bone while one quarter is found in the lungs. With the exception of the lung, all this aluminum is expected to have been absorbed through the gut. Although the literature values are highly variable due to differences in analytical techniques, urinary excretion studies show that the gut absorbs approximately 0.01% of all aluminum ingested [20,10].

Aluminum is clearly neurotoxic. Normal and lethal brain tissue concentrations are documented in many reports [21,22,23]. According to data presented, the lethal dose of aluminum exceeds the normal level by only a factor of 3-10. Estimations of normal uptake of aluminum based upon intestinal absorption studies is consistent with a model by which aluminum is slowly accumulated in the brain and cannot be eliminated. It is interesting to note that, according to this rate of absorbtion and estimated quantities of aluminum ingestion, the average individual would accumulate a lethal aluminum dose (about 4mg/kg wet weight brain tissue) in approximately 100 years.

Aluminum Effects on Proteins: The role of Calmodulin:

Aluminum has been shown to interact with ATP forming a complex more stable than the physiologically useful Mg^{2+} complex [24]. The subsequent competetive inhibition by this complex of enzymes which have an ATP requirement has been proposed as a major mechanism of toxicity [25]. Other enzymes reported to be inhibited by aluminum include: acetylcholinesterase [26,27], adenylate cyclase [28,29], serum cholinesterase, [30], catechol-O-methyltransferase [31], 3',5'-cyclic nucleotide phosphodiesterase [32], and alkaline phosphatase [33,34]. In addition to influencing the above enzymes, aluminum has been reported to have an effect on structural proteins. Bonhaus et al. [35] have reported a destablizing effect of aluminum on microtubule proteins both in cell culture and in vivo. This correlates with reports of neurofibril tangles in the nervous tissue of individuals suffering from various aluminum related neurodegenerative disorders.

The structure and function of the multifunctional regulatory protein, calmodulin, is reportedly altered by toxic aluminum species (appendix A) [32]. Interaction between aluminum and calmodulin results in a reduction in the alpha helix of the protein as monitored by ultra-violet circular dichroism, an increase in the hydrophobic surface area as indicated by the fluorescent hydrophobic surface

probe ANS, and a concomittant decrease in the ability of calmodulin to activate 3',5'-cyclic nucleotide phosphodiesterase. Spacial geometry established between calmodulin and the model target peptide, mastoparan is altered in the presence of aluminum ions (see appendix B). Taken together, this physical and biochemical evidence of aluminum induced lesioning of calmodulin may signal an important toxicological effect of this metal. Partial reversal of the toxic effects of aluminum ions on calmodulin has been documented [36]. Citrate has been shown to prevent complete destruction of the regulatory role of calmodulin in vitro and this organic acid has been proposed as a natural protectant against the deleterious effects of aluminum in maize [37]

Aluminum ions affect the dynamics of living membranes:

It is now clear that there exists some mechanism by which cells and organisms control and adjust lipid composition and order [38,39]. Evidence lies in the distinctive lipid composition of different biological membranes and highly reproducible fluidity determinations among various membranes. Apparently such mechanisms exist to maintain the status quo under conditions which seem to perturb membrane fluitity. Maintainance of fluidity allows physiologically important processes such as motility and activity of

membrane bound enzymes to proceed. Aluminum is reported to decrease membrane fluidity in Thermoplasma acidophilum both in isolated plasma membrane preparations and in living membranes [40]. Aluminum may stiffen the bilayer by displacing calcium and magnesium from the surface of the membrane and effectively crosslinking phospholipid components. Aluminum has also been shown to alter membrane fluidity in plasma membrane vesicles isolated from maize [37] and has recently been shown to disturb proton transport across plasma membrane of the same species [41]. Preliminary experiments conducted on plasma membrane vesicles isolated from trout gill tissue indicate that aluminum may cause membrane stiffening concomitant with reduced ATPase activity in this tissue. Furthermore, alterations in fluidity affects the formation of gap junctions disrupting normal cell-cell communications in cultured glial cells [41]. Calcium influx in these cells is probably disturbed and could be measured with recently developed fluorescent calcium indicators such as fura-2. Whether alterations in calcium influx induced by aluminum are a result of aluminum lesioning of calmodulin or are a function of altered membrane fluidity is an important question. However, disturbances in either cellular component would ultimately be expected to influence the other and further work should be directed toward discriminating between these effects.

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CHAPTER II

THE ROLE OF pH IN MODULATING CALMODULIN CONFORMATION AND FUNCTION

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SHIXING YUAN AND ALFRED HAUG

KEY WORDS: CALMODULIN, CIRCULAR DICHROISM, FLUORESCENCE ANISOTROPY, HYDROGEN ION CONCENTRATION.

When applied to cells, a stimulus may elicit a physiological response where calcium ions act as second messengers. The calcium signal is transmitted to target enzymes and/or structural proteins by modulating calcium-binding proteins. Among these calcium- binding proteins is calmodulin which seems to be involved in the regulation of numerous enzymes and biochemical processes of eukaryotic species (1). Protein conformational changes appear to play a pivotal role in the interaction of calmodulin with target proteins to transmit the message of its specifically bound calcium. This is illustrated by observations that there exists a delicate relationship between the number of calcium ions bound to calmodulin and its ability to bind to the model-target peptide mastoparan (2). Since calmodulin harbors numerous negative electric charges, the association constant of Ca^{2+} increases with increasing pH or decreasing ionic strength. Experiments have also indicated that the cooperativity of the binding sites increases with decreasing pH or increasing KCl concentration (3). At pH values below 3.5, calcium fails to associate with calmodulin (4).

Since fluctuations of intracellular hydrogen ion concentration occur over an order of magnitude in response to hormonal signal- ling and increased metabolic activity (5), it is conceivable that fluctuations in cytosolic pH lead to changes in calmodulin confor- mation. Considering the complexity of the protein's conformational

space and the existence of multiple thermally accessible energy minima (6), pH induced changes in the protein's energy may therefore be of critical importance for calmodulin's multifunctional activities. A pH dependence of calmodulin activity is illustrated by findings that calmodulin binding to brain microsomes, rich in synaptic membranes, exhibited a pH optimum near pH 7.0 (7).

With this in mind, we report in this article on the pH depen- dence of calmodulin conformation and activity of calmodulin which may be important for the regulation of calcium signalling. We present evidence for pH dependent conformational changes in calmodulin which result in altered binding characteristics for model targets and variable activity of calmodulin-dependent 3'-5'- cyclic nucleotide phosphodiesterase.

MATERIALS AND METHODS[1]

Materials. Affi-Gel phenothiazine, AG 1X-8, Chelex-100, and electrophoresis- grade acrylamide were purchased from Bio-Rad Labs. (Richmond, CA). DEAE Sephadex, bovine brain acetone powder, activator-deficient 3'-5'-cyclic nucleotide phosphodiesterase, Tris, Mes[2] and Mops buffers were

[1]Abbreviations: cAMP, adenosine 3':5' cyclic monophosphate; ANS, 8-anilino naphthalene sulfonic acid; CD, circular dichroism; Mes, 2-[N-morpholino] ethane sulfonic acid; Mops, 3-[N-morpholino] propane sulfonic acid.

from Sigma Chemical Co. (St. Louis, MO). Monobromotrimethyl-ammoniobimane was obtained from Calbiochem (La Jolla, CA) under the brand name Thiolyte MQ. 8-anilino-1-napthalene sulfonate (ANS) was purchased from Molecular Probes (Eugene, OR).

Quartz cuvettes (Beckman, Palo Alto, CA) used for spectroscopic studies were of one centimeter path-length and acid-washed in 70% nitric acid then rinsed with glass double-distilled, deionized water. All other materials used were of the highest grade available.

Calmodulin. Calmodulin was isolated from bovine brain or from commercially available spinach according to our laboratory procedures (8,9). Calmodulin isolated by these procedures showed no contamination as judged by the absence of tryptophan emission upon excitation with 295 nm light. Protein concentrations were determined on a Perkin-Elmer Lambda-7 UV/VIS spectrophotometer (Norwalk, CT). The extinction coefficient of calmodulin was taken to be 3300 M^{-1} cm^{-1}(10). The concentration of spinach calmodulin was measured according to the Bradford method (11) with a kit commercially available from Bio-Rad (Richmond, CA).

Spectroscopic experiments. Circular dichroism experiments were performed on a Jasco spectropolarimeter, model ORD/UV/CD-5, modified by Sproul Scientific (Boulder Creek, CA). The instrument was calibrated with a 0.1% solution of d-10-camphor sulfonic acid (Kodak, Rochester, NY) which gave

a 15.6 cm deflection at 290nm corresponding to an observed ellipticity of +7260 mdeg cm^2 $dmol^{-1}$ (12). Spectra were recorded at room temperature between the wavelengths of 260 and 200nm. Buffers for the CD experiments contained 10 mM Tris and were adjusted to the appropriate pH value by addition of one μL aliquots of either 1N HCl or 1N NaOH. pH values were recorded on a Beckman model pH meter prior to and after each measurement. The instrument was calibrated with standard buffer solutions integer pH values as appropriate. Fluorescence experiments were carried out on a model 4800 spectrofluorimeter by SLM Instruments (Urbana, IL). The instru- ment is equipped with Glan-Thompson calcite polarizers and a Debye-Sears modulator allowing sinusoidal modulation of the excitation beam at three frequencies. Polarization measurements were recorded with the instrument in T-format using excitation and emission filters as indicated in figure legends. Steady state fluorescence anisotropy, r, may be defined as (13,14):

$$r=(I_{//}-I_{\perp})/(I_{//}+2I_{\perp})$$

where $I_{//}$ and $I_{\perp}$ refer to the intensity of the emission measured parallel and perpendicular to the plane of the exciting beam, respectively. Anisotropy can

be related to the fluorescence lifetime and average rotational correlation time by the Perrin equation (14):

$$r=r_o(1+\tau/\phi)^{-1}$$

where r_o represents the measured anisotropy in the absence of rotational motion, ϕ is the rotational correlation time.

Fluorescence lifetimes, τ , were calculated from the phase shift and demodulation of the emission intensity. During the lifetime experiments, Di-methyl POPOP in ethanol was used as a mono- exponential lifetime standard (15).

Phosphodiesterase activity. The calmodulin-stimulated 3'-5'-.cyclic nucleotide phosphodiesterase activity was determined according to standard methods. Briefly, the procedure involved incubation of 100 μg of the enzyme, and 1 mM cAMP with 500 μg of bovine brain calmodulin in 20 mM Tris and 3 mM Mg^{2+}. The reaction was allowed to proceed for 10 minutes at 30° C, then was stopped by boiling for 1 minute followed by chilling to 0° C. The mixture was then incubated for 15 minutes with 5'-nucleo- tidase at 30° C followed by colorimetric determination of free phosphate by a previously established method (16). Basal enzyme activity was determined in the presence of 50 μM EGTA.

RESULTS

ANS Fluorescence. The present experiments indicate that the ANS fluorescence intensity of the dye-calmodulin complex (9) generally increases as the pH decreases (Fig. 1). As a control, the fluo- rescence intensity of the free dye remained virtually unaffected by pH changes, consistent with previous data (17). In the presence of saturating calcium concentrations, the pH dependence of the ANS fluorescence intensity of the dye-calmodulin complex appears to be more pronounced than that observed for the ANS-apoprotein complex. These pH-dependent changes in ANS fluorescence probably result from proton-induced modulation of the protein's hydrophobic sur- face because binding of this dye to hydrophobic regions of pro- teins is known to cause a pronounced increase in fluorescence intensity compared with that observed in a polar environment (18). At a fixed pH value, calcium binding leads to the exposure of a hydrophobic surface region which has been implicated as a potential docking region for calmodulin targets (1).

Circular dichroism. The mean ellipticity recorded at 222 nm of a 10uM solution of bovine brain calmodulin is dependent upon hydrogen ion concentration (Fig. 1). In the absence of added Ca^{2+}, between the pH values of 6 and 7, the protein

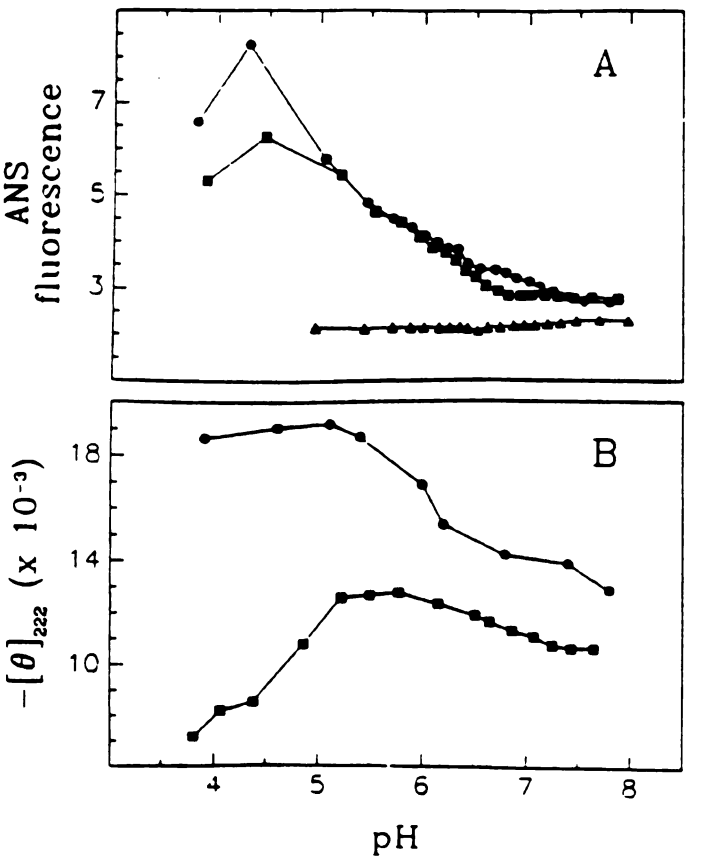

Fig.1. pH dependence of ANS fluorescence intensity at 490 nm (A), and mean ellipticity (B) in units of mdeg cm^2 $dmol^{-1}$ recorded at 222 nm of bovine brain calmodulin. The corresponding physical parameters were measured in the presence (●), or in the absence (■) of saturating calcium concentrations. The concentrations for calmodulin and ANS were 10 μM and 2 μM, respectively, in 10 mM Pipes buffer (A). The pH dependence of fluorescence of free ANS was also determined (▲). In (B) the buffer was 10 mM Tris; pH was adjusted by μL additions of either 1N HCl or 1N NaOH and was recorded prior to and following each measurement.

undergoes a change from -11 000 mdeg cm^2/dmol to about -12,500 mdeg cm^2/dmol. This change can be attributed to increased helical coiling and subsequent exposure of hydrophobic sidechain regions of calmodulin, consistent with data from our ANS experiments. Under saturating calcium conditions, the helicity of the protein is increased across the pH region investigated. Notably different in the case of calcium-calmodulin is the ability of the metal to protect the protein from drastic conformational changes observed at lower pH values in apocalmodulin.

Fluorescence studies. Mastoparan-X (M_r=1556) is a basic, 14 amino acid residue peptide known to bind to calmodulin with high affinity in a calcium-dependent manner and thereby inhibits calmodulin mediated cellular processes (19). Steady state polarization of the single tryptophanyl residue of mastoparan-X was therefore used as a sensitive measure to gain insight into pH dependent association between calmodulin and this model target. Calmodulin associates with mastoparan at a molar ratio of unity (Fig. 2). At a pH value of 7.5, the fluorescence anisotropy attains a maximum value of 0.115. On the other hand, at pH 6.5, the maximum anisotropy is reduced by about 16% indicating an enhanced randomization of the tryptophan emission dipole. Greater randomization may result from an increase in segmental protein motion in the chromophore's interface region with calmodulin. For free mastoparan-X, anisotropy values

of 0.048, and 0.044 (error about 6%) were determined at the pH values of 7.5 and 6.5, respectively.

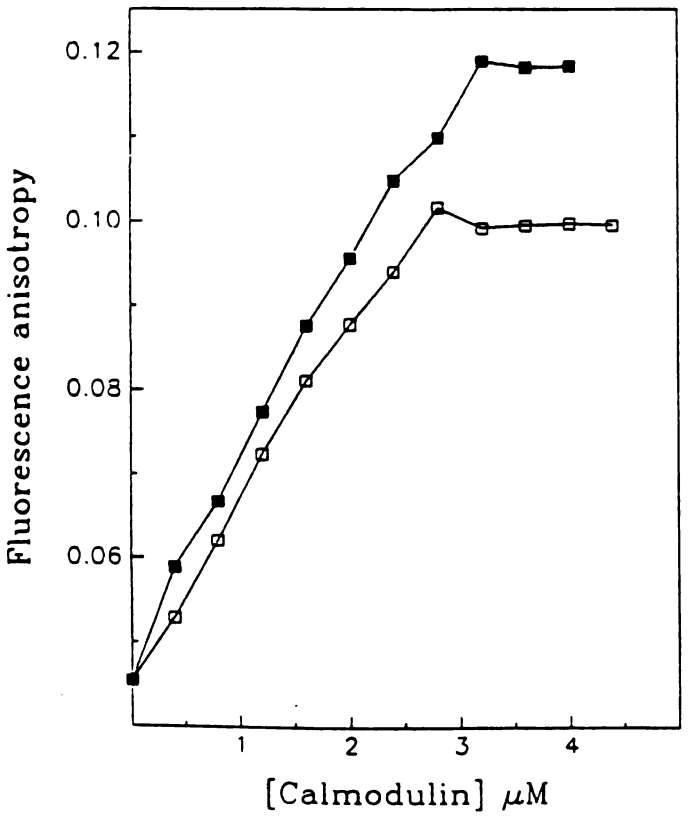

Fig.2. Steady state fluorescence anisotropy of the single tryptophanyl residue of mastoparan-x upon titration with bovine brain calmodulin at pH 7.5 (■) and pH 6.5 (□), respectively. Buffer solution contained 30 mM Mops, 0.2 N KCl and 100 μM calcium. Excitation was at 295 nm through a Corning CS7-54 filter. Emission was recorded in T-format through matching CS0-54 filters. Control experiments indicated the anisotropy of free mastoparan to be relatively pH independent (see text). The error in measurement is approximately 0.002.

To determine pH dependent alterations at a defined site on calmodulin, the single cysteinyl residue 26 (20) of spinach calmodulin was labelled with a thiol-specific bimane fluorescence probe. Control experiments indicated an equimolar ratio of bimane probe bound per calmodulin as measured from the known protein concentration and the probe's extinction coefficient, ε =5,700 at 378 nm (21). Moreover, bimane-labelled spinach calmodulin had practically the same biochemical activity as that of unlabelled calmodulin, as determined by an enzyme assay of calmodulin- and calcium-dependent cyclic nucleotide phosphodiesterase activity.

With bimane-labelled spinach calmodulin, a protein which shares a high degree of homology with bovine calmodulin, motional proper- ties were examined by measuring the fluorescence anisotropy of the dye attached to the single cysteinyl residue of the plant protein (Fig. 3). In Perrin plots the data points obtained were analyzed by linear regression and the resulting straight lines (correlation coefficient 0.998) intersected on the ordinate at r_o^{-1}, the reciprocal limiting anisotropy (14). A value of r_o=0.198 at pH 7.5, indicates a greater degree of protein segmental motion at the probe's site, compared with a value of r_o=0.214, at pH 6.5 (Table I). According to Student's t test, the intercepts on the ordinate are significant within 95% confidence. Enhanced segmental motion at the

higher pH value is also reflected by the steady state anisotropy and the rotational correlation times (Table I).

Calmodulin activity. The ability of bovine brain calmodulin to stimulate cyclic nucleotide phosphodiesterase activity is dependent on the hydrogen ion concentrations (Fig.4). Minimum activity was recorded at 6.5 while maximal activity seems to occur at 7.5. The existence of this pH maximum has been exploited in established procedures to determine calmodulin-stimulated phospho- diesterase activity around pH 8.0 (22), although detailed studies on the pH dependence of this enzyme are apparently lacking. The basal enzymatic activity appears to be relatively insensitive to changes in pH (Fig. 4).

DISCUSSION

Besides knowledge of the well-defined equilibrium configuration of globular proteins, dynamic aspects of structural fluctuations, and their relation to conformational changes also appear to be crucial for an understanding of protein activity (23). Since the protein's potential energy surface is characterized by a multitude of thermally accessible minima (6), binding of small molecules to the macromolecule may readily produce structural alterations which, in turn, affect associated

Table I

Molecular parameters of the bimane fluorescence probe attached to the single cysteinyl residue 26 of spinach calmodulin[a]

pH	τ_p (ns)	τ_m (ns)	r_o	r	ϕ_p (ns)	ϕ_m (ns)
7.5	5.53±0.04	6.55±0.03	0.198	0.087	4.31	5.13
6.5	6.00±0.03	8.60±0.04	0.214	0.094	4.70	6.74

[a]Apparent fluorescence lifetimes, τ_p, and τ_m, were determined from phase shift and demodulation of the emission signal, respectively. The sample was excited at 385 nm with intensity modulation at 18 MHz. ϕ_p and ϕ_m are the average apparent rotational correlation times of the bimane probe as calculated from data obtained with the phase shift and demodulation methods, respectively. r is the steady state anisotropy of the bimane label bound to the protein's thiol group. r_o represents the limiting anisotropy obtained by extrapolation of regression lines (Fig.3). The calmodulin concentration was 6 μM in 10 mM Mops buffer, 100 mM KCl, and saturating Ca^{2+} concentration.

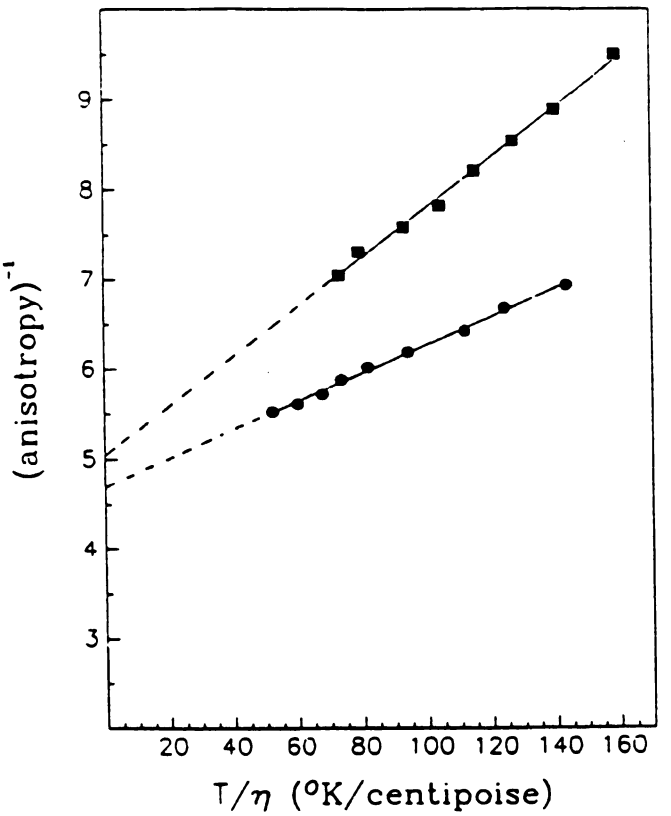

Fig.3. Perrin plot of bimane fluorescence label attached to the cysteinyl residue 26 of spinach calmodulin at pH 7.5 (■) and pH 6.5 (●), respectively. An equimolar ratio of probe-to-calmodulin was established using the dye's known extinction coefficient and and a standard protein assay, respectively. The viscosity was altered by the addition of 100 μL aliquots of stock sucrose (1 gm/ml), mixed with appropriate buffer. Sample solutions contained 2 ml of 6 μM calmodulin in 10 mM Mops and 0.1 N KCl. Excitation was at 385 nm and emission recorded through Corning CS3-72 filters. Extrapolated values of r_o^{-1} are significant by Student's t evaluation.

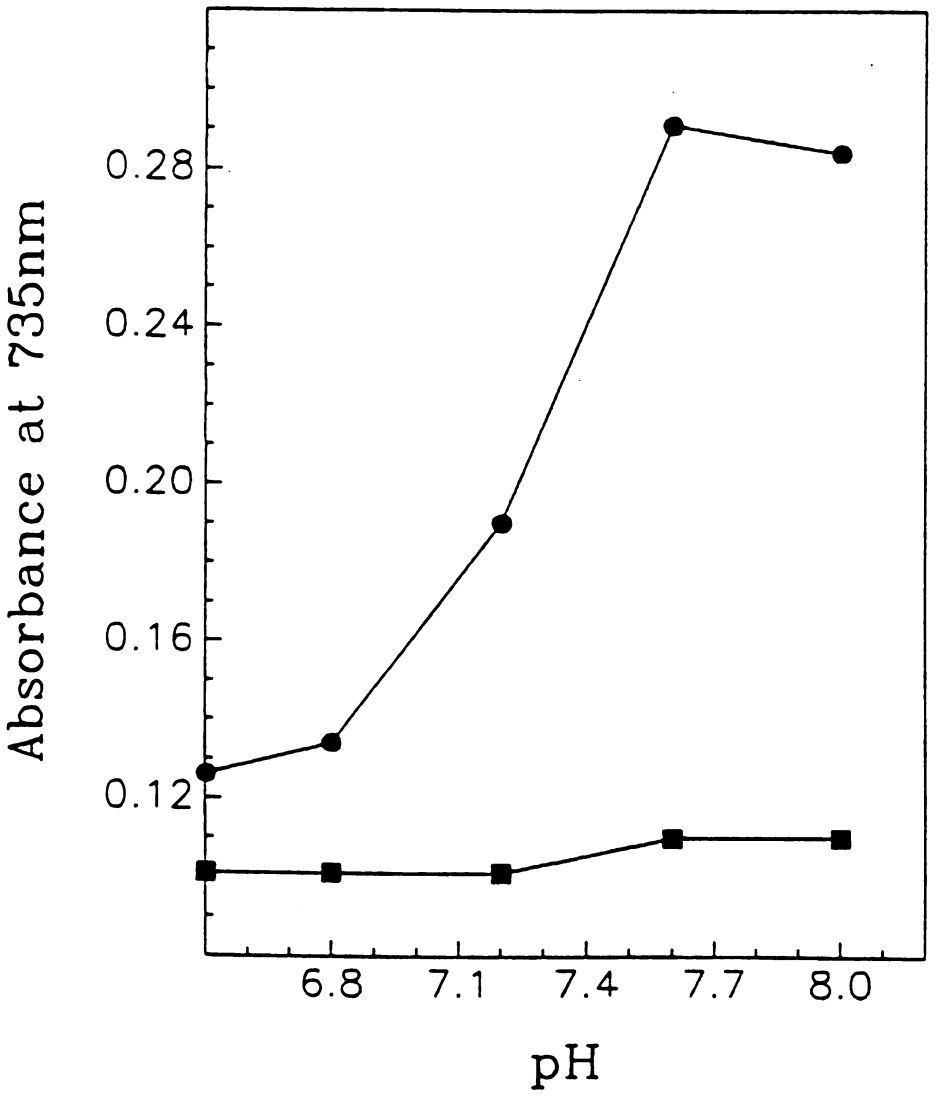

Fig.4. pH dependence of calmodulin-stimulated 3':5'-cyclic nucleotide phosphodiesterase activity (●). The basal enzyme activity was determined in the absence of calmodulin and calcium (■). Solutions contained 100 µg enzyme. 500 ng calmodulin was added to stimulate the enzymatic activity. Absorbance was recorded on a Gilford model 2400.

regulatory activities. With this in mind, one has to expect that lowering the pH also produces structural alterations as a result of a reduction in the electro- static repulsion between charges on the surface of calmodulin which is an acidic protein. pH related structural changes are illustrated by our findings of increased hydrophobic surface exposure and alterations in the mean residue ellipticity which may be an indicator of helices present (24). Furthermore, pH induced changes in electrostatic interactions between charges also influence Ca^{2+} binding to the specific sites in calmodulin. In fact, the apparent association constant for Ca^{2+} binding, K, is dependent on the electric field as follows (25), $K= K_o exp(-4wZ)$. Here, Z is the net charge of the protein, w is a parameter representative of electrostatic interactions, and K_o is obviously the association constant in the absence of an electric field. At neutral pH, the net negative charge is 27 in the apo-protein (26). On the other hand, with calcium bound to calmodulin, the Ca^{2+}- promoted increase in helices seems to stabilize the protein against pH changes, in particular at lower pH values (Fig.1).

pH dependent motional changes are also occurring in the inter- face region of calmodulin with the model target, mastoparan. This amphiphilic peptide has an association constant of 3.3 nM^{-1} with calmodulin (27) and probably binds to the high-affinity region III of

calmodulin's C-terminal half (28), when Ca^{2+} is present. Closely related to mastoparan is melittin whose interface with calmodulin appeared to become more polar as a result of aluminum- induced breakage of helices in calmodulin. In such a polar envi- ronment, the single tryptophanyl residue of melittin also experi- enced changes in motional properties and in access to quenching molecules, compared with comparable parameters characteristic for a more nonpolar microenvironment (29).

The bimane fluorescence probe studied resides at the cysteinyl residue 26 (20) of the dumbbell-shaped calmodulin (30). Compared with motional characteristics of the fluorophore at pH 6.5, at the higher pH value the probe seems to experience faster segmental protein motions as reflected by parameters like anisotropy (Table I). At the higher pH value the electrostatic repulsion between charges on the side chains of the acidic protein is probably enhanced. Consequently the coupling between charged sites may be lowered which, in turn, permits faster segmental motions, as observed at the higher pH value.

In summary, our findings reported here support the possibility that cellular pH changes may produce alterations in calmodulin structure and consequently in the protein's capacity to communicate with targets.

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CHAPTER III

ALUMINUM INDUCED CONFORMATIONAL CHANGES IN CALMODULIN ALTER THE DYNAMICS OF INTERACTION WITH MELTITTIN

CHRISTOPHER WEIS and ALFRED HAUG

KEY WORDS: ALUMINUM TOXICITY, CALMODULIN, CIRCULAR DICHROISM, FLUORESCENCE POLARIZATION, MELITTIN, TARGET ENZYMES

Calmodulin is an acidic, heat-stable protein which is pivotal in mediating a multitude of calcium-dependent physiological processes (1). Upon binding of calcium into specific regions, architectural events take place which are necessary for calmodulin's interaction with target proteins. Because melittin has a calmodulin affinity in the nanomolar range, and since it competes with target enzymes for calcium-calmodulin, this small amphiphilic polypeptide has been proposed as a model for investigating the interaction between calcium-calmodulin and its target proteins (2).

Binding of calcium produces specific structural rearrangements in calmodulin, and therefore the protein's conformation and its efficacy to interface with target proteins may be profoundly altered when toxic metal ions interfere with calmodulin. Indeed, upon application of stoichiometric amounts of solvated aluminum species to micromolar calmodulin solutions, the protein's helical content decreases concomitantly with an enhanced exposure of hydrophobic surface domains (3,4) which, in turn, leads to the inhibition of 3',5'-cyclic nucleotide phosphodiesterase (3) and membrane-bound ATPase activity (5). Although aluminum ions (6), like calcium ions (7), strongly prefer oxygen-containing ligands, there exists evidence which suggests that aluminum ions bound to calmodulin do not displace calcium ions (8). Possible aluminum binding sites are carboxylic ligands at the protein's surface. Contrary to

the aluminum ion, the trivalent terbium ion can displace calcium from its specific sites while maintaining thefunctional integrity of calmodulin (9,10).

These aluminum-induced structural changes in calmodulin may constitute a molecular basis for aluminum toxicity (3,4). This broadly defined syndrome occurs in man where aluminum has been implicated in neurological disorders and in osteomalacia-type diseases (11). In plants, aluminum toxicity represents a serious problem of global proportions because vast regions of the earth suffer from soil acidity which is favorable for aluminum mobilization in soil (12).

To assess further aluminum-induced lesions in calmodulin, we present in this article information on aluminum's impact on the interface between calmodulin and melittin as a model for target proteins. Since fast structural fluctuations appear to play a key role in protein dynamics and catalysis (13), it was our aim to study time-dependent processes in the nanosecond range in relation to aluminum-induced structural changes in calmodulin. Therefore, the fluorescence properties of melittin's single tryptophanyl residue were used as sensitive parameters to derive information on melittin's interface with calmodulin, in the presence and absence of aluminum. With aluminum present, the average microenvironment of the fluorophore is modified, probably resulting from rearrangement of water molecules in the protein's solvation shell.

EXPERIMENTAL: METHODS AND MATERIALS

Materials. Affigel phenothiazine, AG 1X-8, Chelex-100, and electrophoresis-grade acrylamide were purchased from Bio-Rad Labs (Richmond,CA). Bovine brain acetone powder, DEAE Sephadex, Tris, Mes[2] and Mops buffer, were obtained from Sigma Chemical Co. (St. Louis, MO). $AlCl_3$ $6H_2O$ was obtained from Mallinckrodt (St. Louis, MO). All other chemicals were of the highest quality available. All cuvettes and glass ware were acid-washed with concentrated nitric acid and rinsed with glass-distilled water, whereas plastic ware was treated with Chelex-100. Buffers were decontaminated of residual metals by passage through Chelex-100 columns.

Calmodulin. Bovine brain calmodulin was isolated by phenothiazine affinity chromatography as previously described (14). To enhance the purity of the isolated protein, modifications were incorporated into the isolation procedure (15). Calmodulin isolated in this manner was free from tryptophan-containing proteins as judged by fluorescence emission. The isolated calmodulin also activated 3',5'-cyclic nucleotide phosphodiesterase in a calcium-dependent fashion. The protein was further tested for purity with polyacrylamide gel electrophoresis and by an NMR spectrum at 250 MHz. Analysis for aluminum and calcium in the purified protein was performed by measuring the metal content on a polarized Zeeman atomic absorption

spectrophotometer, model 180-80 (Hitachi, Tokyo, Japan). This analysis showed aluminum contamination to be minimal with an aluminum to calmodulin molar ratio of less than 0.1. Calcium content in the purified preparation was less than 0.6 calcium ions per calmodulin molecule. Metal analysis was further confirmed with use of a Jarrell-Ash plasma emission spectrometer, model number 955 Atomcomp. Protein concentrations and ultra violet absorbance scans of calmodulin were determined with a Perkin-Elmer Lambda-7 UV/Vis spectrophotometer (Norwalk, CT). The molar extinction coefficient of bovine brain calmodulin at a wavelength of 277 nm was taken to be 3,300 $M^{-1} \cdot cm^{-1}$ (16). Unless otherwise stated the buffer solutions used contained saturating concentrations of calcium and 0.2 N KCl to minimize possible errors from ionic strength effects (17).

Melittin. Melittin used throughout the experiments described was obtained from Serva Feinbiochemica (Heidelberg, FRG). Purity of the protein was determined by loading 90 μg of the commercial product on a 15 percent polyacrylamide gel. Even at these high loads, the melittin preparation migrated as a single band and showed no apparent contamination with phospholipase A_2. Melittin purchased from this company has also been found to be pure by independent investigation (18). To avoid aggregation of this basic and very hydrophobic protein, all measurements were

conducted at concentrations of less than 50 μM melittin. Below this concentration the protein apparently does not adopt its tetrameric form (19). The molar extinction coefficient of melittin was taken to be 5 470 $M^{-1}cm^{-1}$, at 280 nm (20).

Circular dichroism experiments. Circular dichroic spectra were obtained on a Jasco spectropolarimeter, model ORD/UV/CD-5, which has been modified by Sproul Scientific Instruments of Boulder Creek, CA. For the CD experiments quartz cuvettes with an optical path length of 1 cm were purchased from Beckman Instruments (Palo Alto, CA). The instrument was calibrated with a 10 μM solution of sperm whale myoglobin in aqueous solution at pH 7.0, for which the mean residue ellipticity, $[\Theta]_{222}$, is -25,600 deg cm^2 $dmol^{-1}$ (21). All spectra were recorded at room temperature between the wavelengths of 260 and 210 nm. Buffer solutions for these experiments contained 10 mM Tris adjusted to pH 6.5 with HCl, 0.2 N KCl. Solution volumes were 2 ml and calmodulin concentrations used were 10 μM.

Observed ellipticities of the calmodulin/melittin complex were recorded which are related to the helical content of the protein (22).

Fluorescence studies. Fluorescence experiments were performed on a spectrofluorimeter, model 4800, from SLM Instruments (Urbana, IL). The instrument is interfaced with

a Hewlett-Packard HP-85 desk top computer and plotter to aid in data acquisition and analysis. Quartz cuvettes used were as described above. Sample volumes of 2ml were employed in all the experiments and dilution factors were minimized to 2 percent or less, apart from quenching studies. Polarization experiments involved the employment of Glan-Thompson calcite prism polarizers with high polarization extinction ratio. During the polarization and total intensity experiments the excitation wavelength was 295 nm, slit width 8 nm, and emission was viewed through a Corning CS0-54 ultraviolet light cut-off filter.

Steady sate fluorescence anisotropy, r, is defined as (23):

$$r=(I_{//} - I_1) / (I_{//} + 2I_1)$$

where $I_{//}$ and I_1 refer to the intensity of fluorescence emission measured parallel and perpendicular to the plane of the exciting beam, respectively.

Anisotropy is related to the fluorescence lifetime, τ, and the rotational relaxation time, ϕ, according to the relation:

$$r = r_o(1 + \tau/\phi)^{-1}$$

where r_o represents the anisotropy in the absence of rotational diffusion.

Fluorescence intensity experiments were performed by titrating aliquots of concentrated calmodulin solutions into 10 μM solutions of buffered melittin. Emission scans from 300 to 440 nm were recorded.

Fluorescent lifetimes were calculated from phase shift and demodulation data obtained with a Debye-Sears modulator (24). All fluorescence studies were performed on sample which had an optical density of less than 0.1 at 280 nm, thus scattering corrections were negligible.

The exciting beam was sinusoidally modulated at a frequency of 30 MHz. The modulating tank contained a 19 percent ethanol/81 percent water (v/v) mixture which was changed regularly to keep the ethanol/water ratio constant. 2,5-diphenyl-1,3,4-oxadiazole (Sigma Chemicals, St.Louis, Mo.), a commonly used laser dye, served as a mono-exponential lifetime standard (25). All lifetime experiments were conducted with the emission polarizer rotated to approximately 57 degrees. During the lifetime experiments the excitation wavelength was 295 nm while emission was measured at 340 nm. Applying the techniques described above, our measurements of the fluorescent lifetime of N-acetyl-tryptophanamide in 0.1M sodium phosphate, at pH 7.0, gave a value of 3.0± 0.1 ns, which is in close agreement with the reported value of 3.0 ns (23).

The collisional quencher acrylamide (23) was used to vary the fluorescent lifetime of tryptophan during the lifetime and quenching experiments. 8 M stock solutions of acrylamide contained 0.2N KCl and were adjusted to pH 6.5 to maintain homogeneity of the quenching solution. Each data point reported is the average of 200 individual readings.

RESULTS

Circular dichroism. The observed ellipticity, at 222 nm, of the melittin-calmodulin complex is dependent upon the presence or absence of aluminum ions (Fig.1). Upon titration of melittin onto a 10 μM solution of calmodulin, protein association occurs which is reflected by a biphasic plot. As indicated by a well-defined break in slope, a 1:1 complex is formed between melittin and calcium-calmodulin, consistent with previous reports (20). NMR studies of the interaction between calmodulin and melittin have clearly shown that the increased helicity associated with complex formation may be attributed to coiling in both calmodulin and its target (26,27).

Upon association of aluminum with calcium-calmodulin, normal targeting is altered as evidenced by a reduced total α-helical content. In the unaltered complex total helicity rises to a value of about -65 mdeg at a molar ratio of unity as melittin is titrated onto calcium saturated calmodulin. However, the association of aluminum with calmodulin, still under saturating calcium conditions, results in reduced helical increase in the overall complex. binding of aluminum to calmodulin thus results in structural alterations which impair melittin's association with the regulatory protein, and therefore less helices are formed in the complex.

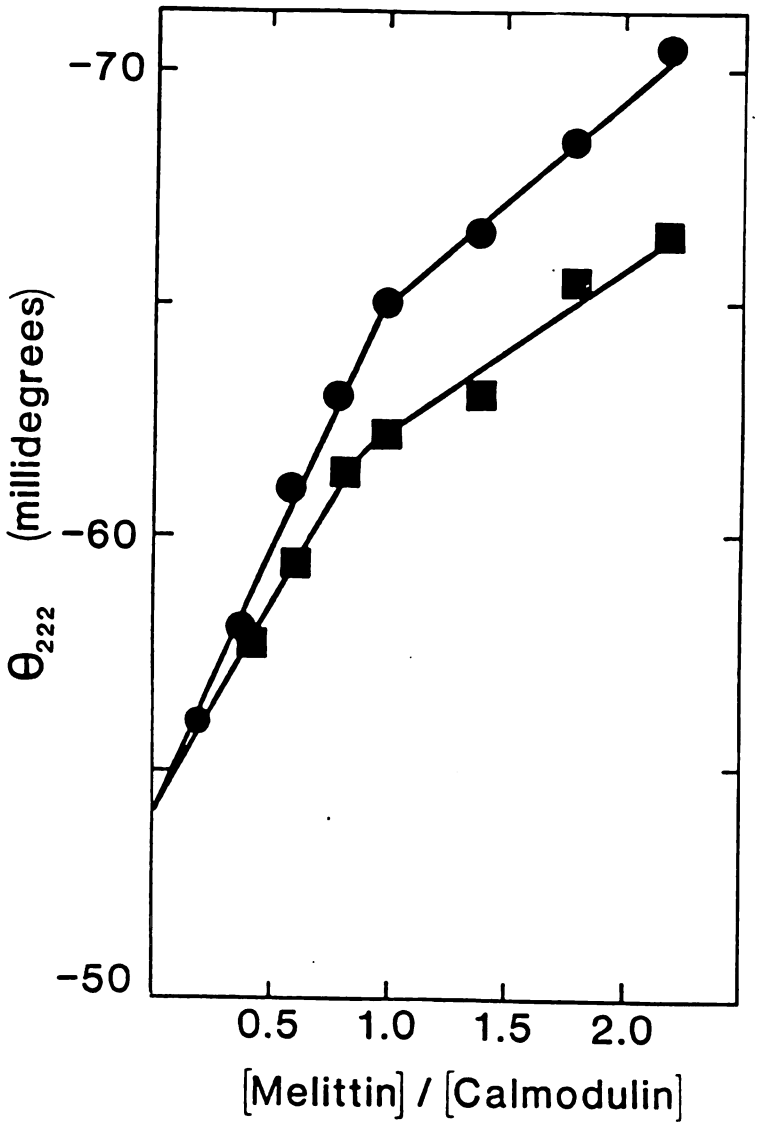

Fig. 1. Titration of 10 μM solutions of calmodulin (●) and aluminum-calmodulin (4:1) (■) by melittin as observed by ultraviolet circular dichroism. The buffer contained 10 mM Tris-HCl, pH 6.5, 0.2 N KCl, and 80 μM $CaCl_2 \cdot 2H_2O$. The abszissa indicates molar ratios. The contributions of calmodulin or aluminum-calmodulin to the ellipticity have been corrected for.

Control experiments indicated that application of an eightfold excess of aluminum ions over melittin alone does not change the protein's CD spectrum. Since the binding affinity of calcium-calmodulin for melittin is reportedly in the nanomolar range (20), ellipticity changes arise from the melittin- calmodulin complex at the micromolar concentrations of both proteins used. We therefore assume that any direct aluminum- induced spectral changes originate from metal interaction with calmodulin rather than melittin.

Quenching of Fluorescence. Acrylamide quenching of tryptophan fluorescence in the calmodulin/melittin complex is found to be altered if aluminum is associated with the calmodulin molecule (Fig.2). The linearity of the plot suggests the existence of a single class of fluorophores accessible to the quenching molecules. Values for the bimolecular quenching constant, k_q, calculated for the quenching of melittin's tryptophanyl residue, when the protein is associated with the aluminum-calmodulin and native calmodulin, both in the presence of calcium, are listed in Table I. As opposed to melittin association with calmodulin, upon association with the aluminum-calmodulin complex, melittin's tryptophanyl residue is apparently more accessible to the quencher as evidenced by the somewhat higher value of k_q. That the quenching of tryptophan with acrylamide is a collisional

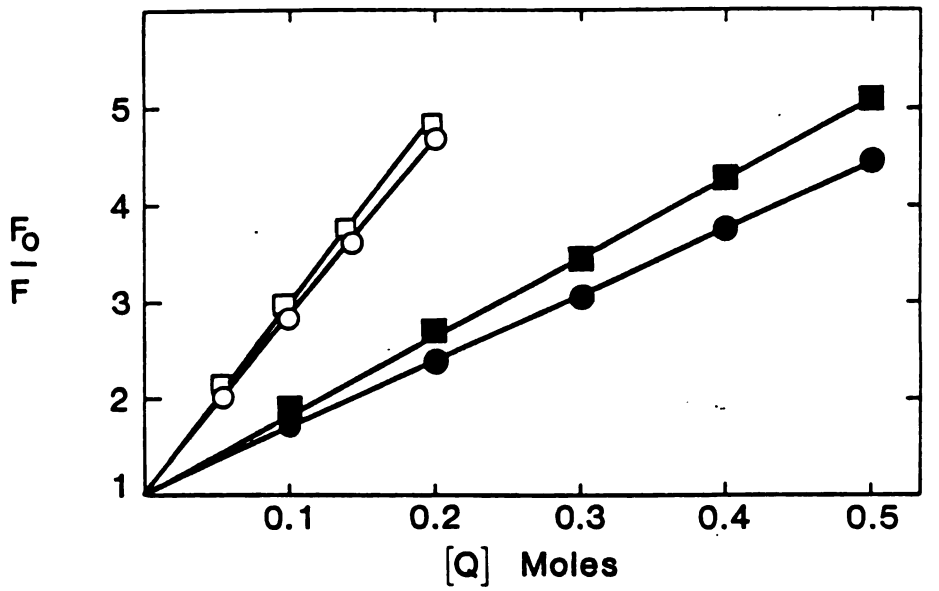

Fig. 2. Fluorescence quenching yield, F_o/F, of melittin's single tryptophanyl residue in the melittin-calmodulin complex. When melittin is associated with calmodulin (●), the fluorophore seems to be more sheltered from the acrylamide quencher, Q. The fluorophore is more accessible to the quenching molecule (■) when melittin associates with aluminum-calmodulin (4:1). (□) and (○) reflect fluorescence quenching of the tryptophanyl residue in free, monomeric melittin (10 μM), in the presence and absence of 40 μM $AlCl_3 \cdot 6H_2O$. The buffer contained 45 mM Mops-KOH, pH 6.5, 0.2 N KCl, 80 μM $CaCl_2 \cdot 2H_2O$. The calmodulin concentration was 10 μM. Excitation wavelength was 295 nm, emission was recorded at 340 nm. F_o and F represent the fluorescence intensities in the absence and presence of quencher molecules.

rather than a static process becomes further evident from lifetime measurements which clearly show that τ_0/τ is not unity.

Fluorescence Intensity Experiments. Upon titration of 5 µl aliquots of calcium saturated calmodulin onto solutions of 10^{-5}M melittin, a dramatic increase in tryptophanyl fluorescence intensity was observed (Fig.3). This increase in the fluorescent intensity, measured at 340 nm, was approximately 17 percent, compared with that in the absence of calcium- calmodulin. In addition, the fluorescence wavelength maximum shifted towards the blue, viz., from 355 nm, with no calmodulin present, to 345 nm, at a molar ratio of 3 calmodulin molecules per melittin. By contrast, when calmodulin, bound to aluminum at a molar ratio of 1:4, was titrated into a solution of melittin, no significant increase in fluorescent intensity was observed and the blue shift in the maximum emission wavelength was less, viz., from 355 nm to 348 (Fig.3), in the presence of saturating calcium concentrations. Since tryptophan emission yields seem to be high in helical portions of a protein (28), the insignificant fluorescence enhancement is probably related to the lower helix content when melittin is associated with aluminum-calmodulin as compared with that of the complex in the absence of aluminum. The fluorescence maximum observed at 355 nm corresponds to that expected for tryptophan

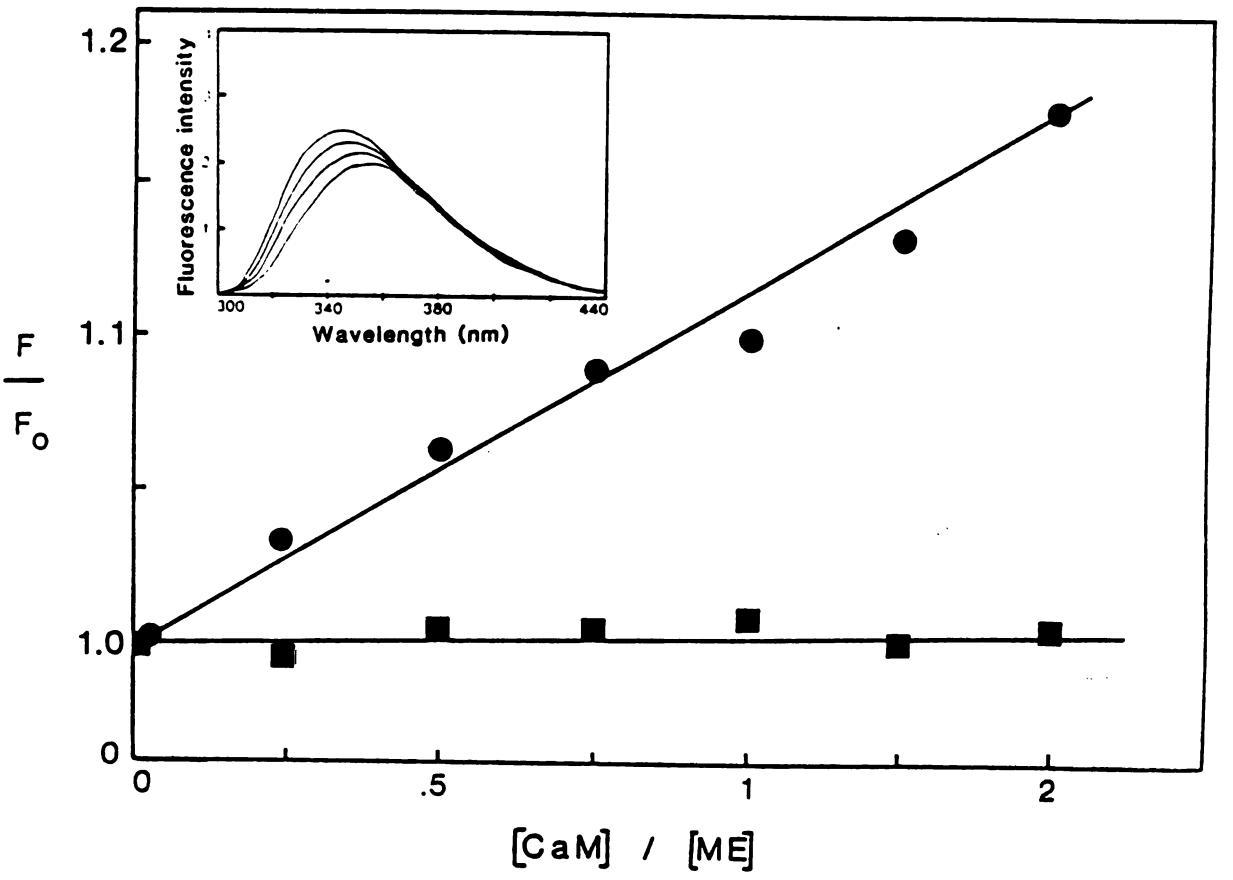

Figure 3. Fluorescence intensity data of a 10 μM solution of melittin upon addition of concentrated aliquots of calmodulin; dilution effects could be ignored. Excitation wavelength was 295 nm. The buffer contained 45 mM Mops-KOH, pH 6.5, 0.2 N KCl, and 80 μM $CaCl_2 \cdot 2H_2O$. Titration of calcium calmodulin (●) and aluminum damaged calmodulin (■). Inset shows emmision spetral blue shift and intensity increase associated with subsequnt titrations of calcium calmodulin.

completely exposed to water (29). Presumably the tryptophanyl residue of melittin is hindered from partitioning into the more hydrophobic areas of the complex when aluminum ions are bound to calmodulin. Alternatively, the diminished blue-shift in the fluorescence maximum of the tryptophanyl residue of melittin in association with aluminum- calmodulin may also arise from melittin being in a less rigid microenvironment (30), compared with the complex in the absence of aluminum ions.

Observation of the polarization anisotropy of the tryptophanyl residue in melittin, when the peptide is bound to calmodulin, gives further indication that aluminum ions alter the association process. Figure 3 shows the anisotropy of the calmodulin- melittin complex to be about 0.11 in the presence of saturating calcium concentrations. Subsequent titration of aluminum ions onto the complex results in a dramatic decrease in the anisotropy at a molar ratio of 2 aluminum ions per complex to a new value of about 0.06. Alteration in the lifetime of fluorescence, possibly due to modification of the hydration structure, or a dramatic increase in the rotational rate of the fluorophore, would be expected to cause such a decrease in the anisotropy.

Rotational correlation time. When melittin is bound to calmodulin, the rotational correlation time of the tryptophanyl residue may be calculated from results derived from measurements

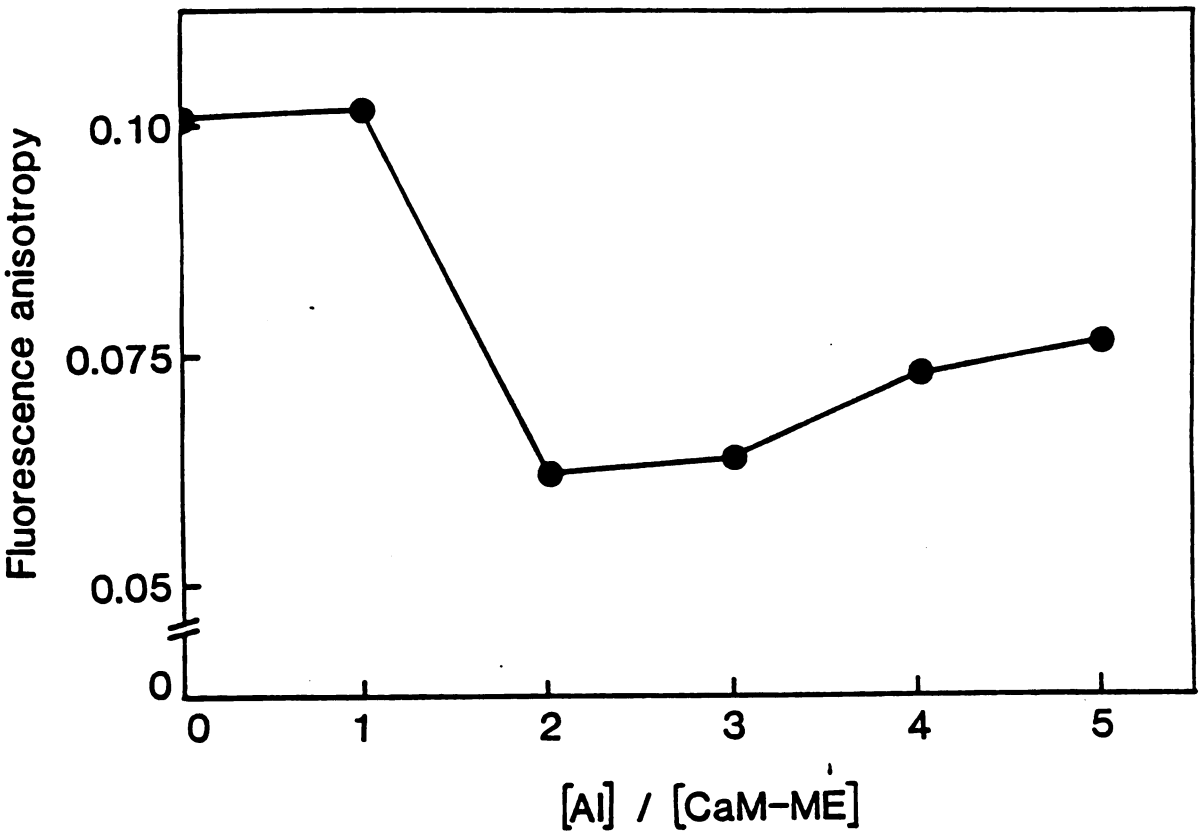

Figure 4. Fluorescence polarization of the melittin-calmodulin complex (9 μM) upon titration with aluminum. Excitation wavelength was 295 and emission was monitored at 340nm through a Corning CS-054 filter. Buffer was 45mM MOPS, 0.2 N KCl, pH 6.5, and 80 μM $CaCl_2 \cdot 2H_2O$. Error in polarization about 10 percent.

of fluorescence anisotropy vs. fluorescence lifetime (Fig.5). The respective data points define a slope when analyzed by linear regression. Extrapolating the slope to the ordinate of the graph, a value for the apparent limiting anisotropy, r(0), can be derived. The slope is equal to $(r(0)\phi_A)^{-1}$, from which the apparent rotational correlation time, ϕ_A, can be determined (31).

The values of the apparent limiting anisotropy, r(0), are smaller than the value of the limiting anisotropy of tryptophan, r_o, determined in the absence of rotation (31). In the presence of aluminum, the apparent limiting anisotropy, r(0), is larger than that observed with aluminum absent (Table I). This increase suggests that the motional freedom of the tryptophanyl residue is somewhat restricted when melittin is associated with aluminum- calmodulin. Conceivably tryptophanyl residues are involved in hydrogen bonding (32). Moreover, the lifetime and the rotational correlation time of the tryptophanyl residue is smaller when melittin is associated with aluminum-calmodulin (Table I). Compared with the tryptophanyl residue in the absence of aluminum, a shortening of the fluorophore's lifetime and rotational correlation time may result from aluminum-induced changes in the fluorophore's average microenvironment. This notion is supported by our observations on the dramatic decrease of tryptophan fluorescence and the smaller blue-shift in the absorption spectrum when melittin is

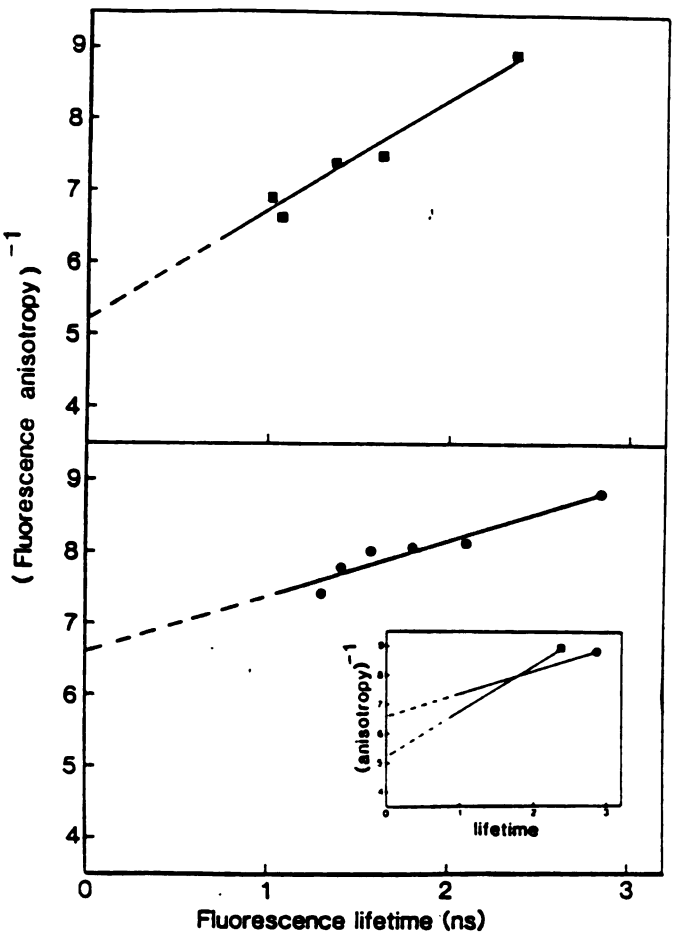

Fig. 5. Fluorescence anisotropy as a function of the lifetime of melittin's tryptophanyl residue upon association of melittin with equimolar calmodulin (top graph), or aluminum-altered calmodulin (4:1) (bottom graph). Inset shows slope relationship between graphs. The protein concentration was 10 μM each. The buffer consisted of 45 mM Mops-KOH, pH 6.5, 0.2 N KCl, and 80 μM $CaCl_2 \cdot 2H_2O$. The excitation wavelength was 295 nm, anisotropic emission was observed at 340 nm on a SLM spectrofluorimeter, model 4 800.

Table I

Fluorescence lifetimes, τ, bimolecular quenching constants, k_q, Stern-Volmer quenching constants, K_D, rotational correlation times, ϕ_A, of melittin's tryptophanyl residue upon association with bovine brain calmodulin, in the presence and absence of aluminum. All experiments were performed in the presence of calcium at a molar ratio of 8:1 for [calcium]/[calmodulin], at pH 6.5, 25°C. Aluminum was present at a molar ratio of 4:1 for [aluminum]/[calmodulin]. The uncertainty of lifetime measurements is approximately 0.1 ns.

MELITTIN-CALMODULIN	τ ns	$k_q 19^{-9}$ $M^{-1}s^{-1}$	K_D M-1	ϕA ns
aluminum absent	2.84	1.20±0.1	3.41±0.05	8.42
aluminum present	2.39	1.62±0.1	3.88±0.05	3.54

associated with aluminum- calmodulin as compared with corresponding results from experiments when aluminum was absent.

DISCUSSION

Binding of aluminum to calmodulin results in profound changes in the protein's helix content and its biochemical activities (3,4). These aluminum-induced structural alterations in calmodulin have repercussions on the protein's capability to interact with target proteins such as melittin described in these studies. This small amphiphilic protein of molecular weight 2 846 comprises 26 amino acid residues, 6 of which are positively charged at neutral pH and 10 are highly apolar (33). The association of basic melittin with acidic calmodulin probably involves electrostatic forces between charged groups (34). Additional stabilization of the melittin complex is provided by hydrophobic interactions. In the presence of calcium, with aluminum absent, calmodulin forms a mononuclear, high affinity complex with melittin, with a dissociation constant in the nanomolar range (20). A nanomolar dissociation constant was also found for a highly basic peptide region which lies near the C-terminal end of skeletal muscle myosin light chain kinase and which binds to calcium-calmodulin (35). As far as melittin's tryptophanyl residue is concerned, proton NMR experiments performed on the calmodulin/melittin complex indicated that the fluorophore

is being shielded from the aqueous environment (27).

However, the type of interaction appears to be altered when melittin associates with aluminum-calmodulin as opposed to native calmodulin. In the presence of aluminum, helix induction in the complex is reduced presumably as a result of weaker hydrophobic interactions. This is supported by findings that a strong enhancement of melittin's helix content is brought about by interactions of melittin with hydrophobic structures such as membranes and proteins (20). Weaker hydrophobic interactions in the complex of melittin with aluminum-calmodulin are also indicated by the smaller blue-shift in the tryptophan maximum as opposed to that of the melittin-calmodulin complex, in the absence of aluminum. Furthermore, the observation of a shorter fluorescence lifetime and a lower fluorescence intensity are also consistent with the existence of a more polar microenvironment around the fluorophore in the presence of aluminum because tryptophanyl residues exposed to water generally display shorter lifetimes and smaller quantum yields (36). Apparently the blue-shift is not a result of a rigid microenvironment around the fluorophore.

The notion that the fluorophore is in a more polar micro- environment in the presence of aluminum is in accord with findings that aluminum-calmodulin has a more open structure as compared with the more compact structure of

calmodulin (3,4). For example, aluminum breaks helices, and aluminum-calmodulin migrates slower in electrophoresis studies. Aluminum-induced breakage of helices necessarily leads to a rearrangement of water molecules. The more open structure of aluminum-calmodulin presumably has a solvation structure different from that of calmodulin. The existence of a more open structure would be favorable for access of quenching molecules to melittin's tryptophanyl residue which is in accord with our observations. Furthermore, an increased presence of water molecules in aluminum-calmodulin would be less favorable for helix formation in melittin and there exists the possibility that these water molecules may participate in forming hydrogen bonds with the tryptophanyl residue. This is consistent with our interpretation of the enhanced value of r(0) relative to that extrapolated from data obtained from experiments performed in the absence of aluminum.

In the more open structure of aluminum-calmodulin, as opposed to that of calmodulin, the rotational correlation time of melittin's fluorophore may become faster as a result of changes in its average microenvironment. It seems worth noting that a spin probe attached to aluminum-calmodulin is less immobilized than that covalently fixed to calmodulin in the absence of aluminum (37).

It is tempting to relate our results on the rotational correlation times to the three-dimensional structure of

calmodulin (38). The calmodulin molecule has the shape of a dumbbell consisting of two globular lobes connected by a long exposed α-helix, where each lobe contains two calcium-binding domains. Let us further consider the overall rotational corre- lation time of a hydrated spherical molecule according to the Einstein-Stokes equation. For a combined molecular weight for calmodulin with melittin, M = 20,000, a partial specific volume of 0.73 cm^3/g and a typical hydration of 0.2, a rotational correlation time of about 8.1 ns can be calculated. On the other hand, the rotational correlation time for the calmodulin/melittin complex, in the absence of aluminum, was found to be 8.42 ns. Using this simple model, melittin's tryptophanyl residue has little internal freedom, since the protein complex seems to rotate as a whole. This interpretation appears to be consistent with recent findings that mastoparan, a small peptide similar to melittin, binds to residues between sequence positions 73 and 106 of calmodulin (39). Such a location may be favorable for inter- lobe communication in the presence of proper targets. In the absence of target proteins, calmodulin seems to have a fairly flexible structure (40).

Conducting experiments in the presence of aluminum ions, we measured a rotational correlation time of 3.54 ns (Table I). A large increase in segmental motion of the complex

apparently occurs in the vicinity of the tryptophanyl residue and we attribute this increase to the helix breaking properties of the toxic aluminum species. While it might be intriguing to speculate that aluminum decouples in part interlobe communication, the experimental data base to support such a notion is currently not available.

Assuming the applicability of the Einstein-Stokes equation, free, hydrated melittin would have a correlation time of about 1.1 ns. However, in our experimental set-up we cannot resolve correlation times which contribute to the average time and may in part arise from a subpopulation of unbound melittin. Attempts to determine binding constants from fluorescence data were unsuccessful because of the low protein concentrations needed for accurate measurements. Usage of melittin labelled with radioactive external tags, such as acetyl groups, would also lead to distorted estimates of binding parameters.

In summary, as deduced from measurements of steady-state and time-dependent processes of melittin interaction with aluminum- calmodulin, the latter structure and associated structural fluctuations differ appreciably from those of melittin- calmodulin. Since structural fluctuations play a crucial role in protein dynamics and catalysis (13), critical interactions are modified to such an extent in aluminum-calmodulin that a proper fit of calmodulin with

target proteins cannot take place. This aluminum-induced mismatch, in turn, may be instrumental in malfunctions of calcium and calmodulin-dependent processes when aluminum ions enter the cell.

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CHAPTER IV

ALTERED MEMBRANE DYNAMICS IN HUMAN RED BLOOD CELL WHITE GHOSTS INDUCED BY ALUMINUM

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KEY WORDS: ALUMINUM TOXICITY, ERYTHROCYE MEMBRANE, FLUORESCENCE POLARIZATION

INTRODUCTION

Aluminum has been implicated as a player in a variety of disorders in man and animals. Among the more well defined of diseases associated with this metal are dialysis related disorders such as osteomalacia, dialysis encephalopathy and microcytic anemia (1,2). Further environmental acidification promises to increase the concentration of mobile aluminum leaching into soil and aqueous environments further reducing crop production and interfering with aquatic life (3).

The high degree of reactivity expressed by aluminum and its abundance in the natural environment combined with its apparent uselesness to living systems indicate this metal as a potential toxin. Proposed mechanisms for its toxicity include inhibition of numerous enzyme systems including hexokinase (4), Na^+-K^+ ATPase (5), and adenylate cyclase (6). The ion has been shown to associate with DNA (7) and cause alterations in microtubules and neurofilaments (8,9). Recently toxic species of aluminum have been shown to alter the dynamic interactions between the calcium regulatory protein, calmodulin and model targets (10)

In blood plasma, aluminum is transported bound to transferrin (11). In individuals with moderately high serum concentrations of aluminum, the ultrafilterable fraction is reported to be about 10% (12). Since, in these individuals, the binding capacity of transferrin could be expected to be

exceeded, aluminum must be transported associated with other available ligands including membranes of cellular blood components. In this study we present evidence for aluminum association with isolated red blood cell membranes and the physical alterations which may result from this association. As judged by sensitive fluorescence measurements conducted with the membrane probe 1,6-diphenyl hexatriene (DPH), lipid packing is increased resulting in reduced membrane fluidity as indicated by time resolved motional dynamics of the probe. Physiological consequences of altered lipid dynamics may result in impaired transmembrane communication, and may translate to altered cellular metabolism and cell structure.

METHODS

Red blood cells were obtained from a local American Red Cross blood bank and white ghosts used in fluorescence studies were prepared immediately by the method of Steck and Kant (13). Briefly, the proceedure involved cell lysis in hypotonic buffer (5mM sodium phosphate, 1mM $MgSO_4$, pH 8) followed by repetitive washing and sedimentaion by centrifugation at 2300g for 10 minutes to remove hemoglobin and contaminating proteases. The membranes were then resealed by warming them for 40 minutes at 37° C in isotonic saline (150 mM NaCl, 5mM HEPES, 100μM Ca^{2+}, pH 7)

and used immediately for experiments.

Membranes were labeled with the fluorescence polarization probe 1,6-diphenyl hexatriene (DPH) which was obtained from Molecular Probes (Eugene, OR). Stock solutions of the probe contained 1mg/ml in tetrahydrafuran and were stored dark at -10° C. Experimental solutions were prepared by adding 100 uL of white ghost membranes to 3mL of isotonic saline solution. 0.5 μL of probe stock solution was introduced and polarization studies began following probe incorporation as judged by stablization of fluorescence intensity. A progressive increase in the fluorescence intensity of the probe occurs as the molecule partitions into the more lipophilic environment of the membrane (14).

Polarization experiments were conducted on an SLM Instruments model 4800 spectrofluorometer (Urbana, Ill). The instrument is equiped with Glan-Thompson polarizers with high polarization- extinction ratio and uses a Hewlett Packard model HP-85 for data analysis. The instrument was operated in T-format throughout the experiments described and matching Corning filters (#3060) were used in the emission windows. To avoid trivial depolarization effects due to scattered light a broad band excitation filter (Corning 5860) was used in the excitation path. For all fluorescence experiments the excitation wavelength was 360nm.

Fluorescence anisotropy is indicative of lipid order according to the following relationship (15):

$$r_{\infty} = {}^{9}/_{8} r_s - {}^{1}/_{20}$$

$$r_{\infty} = {}^{2}/_{5}\, S_p{}^2$$

Where r_{∞} is the limiting fluorescence anisotropy derived from steady-state anisotropy of the rod shaped probe molecule aligned normal to the plane of the bilayer. The molecule does not decay to an isotropic state during the lifetime of the fluorescence. Rather, its motion is hindered by the orderly environment of the acyl chains. r_s is the steady-state anisotropy defined as (16):

$$r_s = (I_{//} - I_{\perp})/I_{//} + 2I_{\perp})$$

Where $I_{//}$ and $I_{\perp}$ refer to the intensity of the emission measured parallel and perpendicular to the plane of excitation respectively. Differential tangents, rotational rates and values for the limiting anisotropy of the diphenylhexatriene molecule were determined using differential polarized phase fluorometric methods as previously described (17). Lifetimes were measured with a circular modulation frequency of 18MHz and were calculated

using dimethyl-POPOP as a monoexponential lifetime standard. Heterogeneity analysis of the fluorescence lifetime indicated that greater than 95% of the recorded value was due to a single component. Temperature was controlled using a Neslab thermostated water bath, model RTE-8, and was continually monitored (± 0.1°C) within the sample solution using an Omega digital thermometer, model 410B-T. Cooling scans were run using a temperature program of 0.5°C/minute to allow for membrane equilibration.

RESULTS

Effects of aluminum on erythrocyte membrane structure and dynamics was judged by steady state and time resolved fluorometric measurements. Titration of metal ions onto dilute solutions of isolated white ghost membranes causes profound changes in the microenvironment of the fluorescent membrane probe DPH as judged by fluorescence anisotropy (fig 1). Aluminum is known to compete with calcium for membrane sites (18) and the subsequent rigidification may constitute an initial mechanism in aluminum intoxication. The temperature dependence of the lipid structural order parameter was calculated from steady state fluorescence anisotropy data to further assess the impact of micromolar quantities of aluminum on lipid packing (19,20). Across the temperature region investigated, 20 μM aluminum

causes increased lipid ordering and an altered phase transition temperature indicative of rigidification (fig 2). Error in the anisotropy was negligible and therefore not reported. Measured values of fluorescence anisotropy remained the same following dilution ruling out the possibility of trivial depolarization due to scattered light. Cautious interpretation of lipid order parameter limits interpretation of the above experiments to lipid packing and allows little interpretation of probe rotational dynamics (21). Time resolved experiments were therefore performed to gain further insight into motional characteristics of the DPH molecule.

Rotation of the DPH molecule is thought to be confined by the lipid environment to a potential energy region defined by the cone angle, θ_c, (table 1). The cone angle is related to the time resolved limiting anisotropy and the anisotropy in the absence of molecular motion (22). Completely unhindered rotation yields a cone angle of 90°. In these experiments the addition of aluminum ions at a concentration of 20 μM caused a reduction in the cone angle of probe rotation corresponding to a temperature change of about 2°C. Such a change would be expected to alter kinetics of membrane associated enzymes and transport proteins (21). In the lower temperature region the average probe rotational rate is severely altered by the presence of micromolar amounts of aluminum (fig 3B).

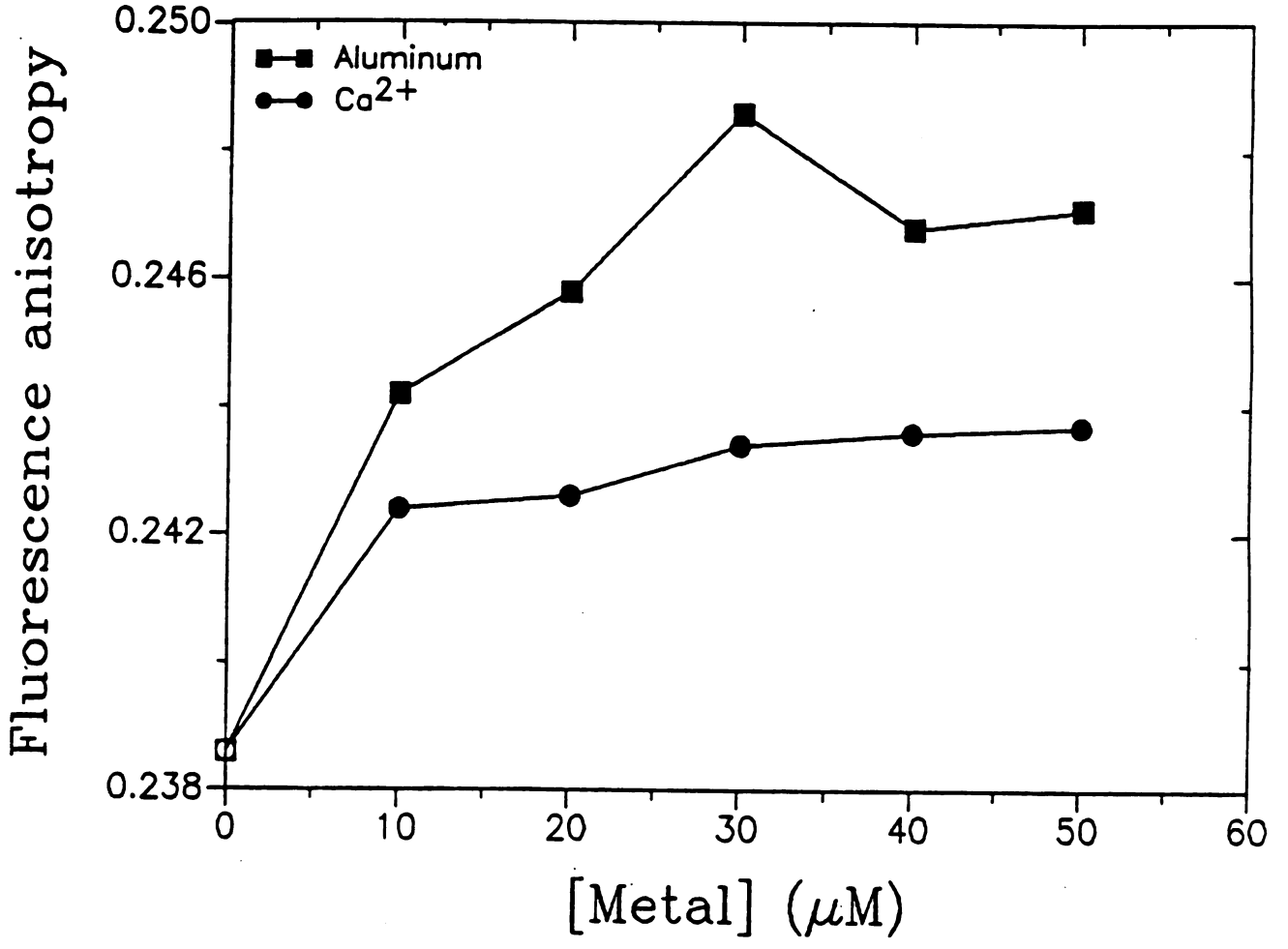

FIGURE 1.

Titration of calcium ions (●) or aluminum ions (■) onto resealed white ghost membranes increases the anisotropic emission of the fluorescent membrane probe DPH. Excitation wavelength was 360 nm and emission was recorded through corning sharp cut filters (number 3060) with the SLM model 4800 spectrofluorometer in T-format. Buffer contained 10mM HEPES, 150mM NaCl, 100μM $MgCl_2$, pH 7.0.

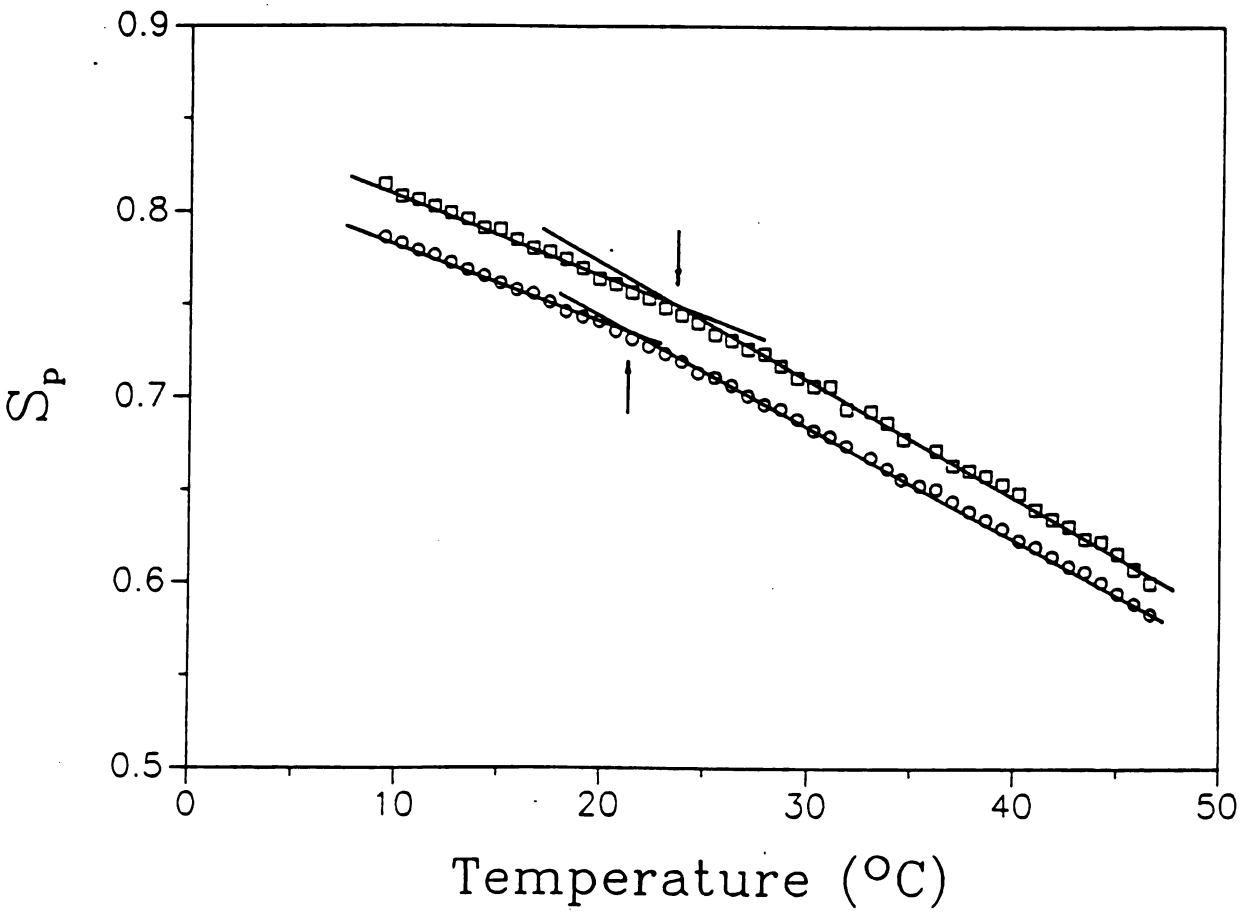

FIGURE 2.

Temperature dependence of the lipid order parameter (S_p) of resealed white ghost membranes as calculated from steady-state fluorescence anisotropy data for DPH. Open squares indicate the presence of 20μM aluminum. Buffer systems for both experiments contained 10mM HEPES, 150mM NaCl, 100μM $MgCl_2$ and 100μM $CaCl_2$. Anisotropy was recorded on an SLM model 4800 spectrofluorometer as described in methods.

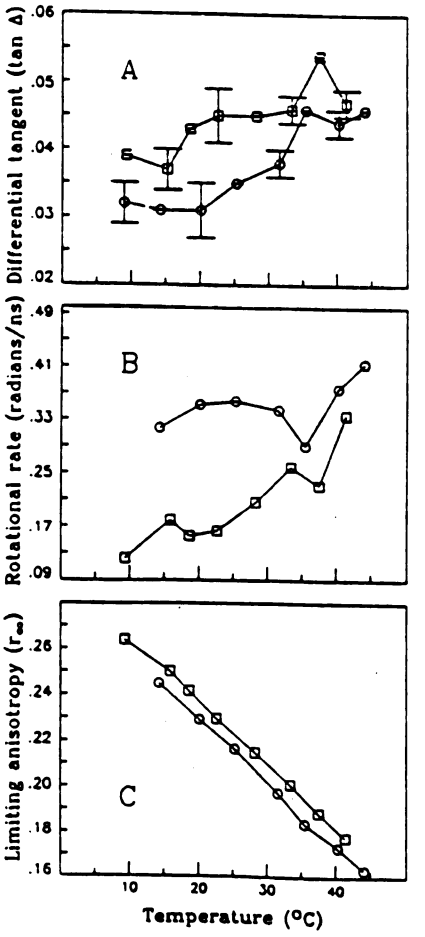

FIGURE 3.

Temperature dependence of time resolved fluorescence parameters recorded from DPH while the probe was imbedded in resealed white ghost membranes. Open squares denote the presence of 20μM $AlCl_3$ in A through C. A circular modulation frequency of 18 MHz was used for the excitation beam. The error in rotational rate (B) is approximately ±0.05 while the error in limiting anisotropy (C) is negligible and therefore could be ignored. Buffers and instrumentation are as previously described.

TABLE 1

Temperature dependence of the rotational cone angle (θ_c) in degrees for the DPH molecule imbedded in resealed white ghost membranes in the presence and absence of 20μM $AlCl_3$.

Incubation temperature	10	20	30	40
θ_c +20uM Al	37.4	40.4	43.6	46.4
θ_c -Al	38.4	41.5	44.3	47.1

Constraints placed on the probe by a less fluid or more orderly arrangement of membrane lipids would be expected to cause such changes in θ_c and rotational rate. Further evidence for increased membrane stiffening or lipid order induced by aluminum is provided by the time resolved limiting anisotropy (fig 3C). In good agreement with the steady state derived values, rotational constraints are indicated across the temperature region observed by a nearly uniform increase in this parameter.

DISCUSSION

These experiments provide further evidence that aluminum binds to and alters lipid structure and dynamics as judged by steady-state and time resolved fluorescence of the membrane probe DPH. Previous experiments have indicated that aluminum ions may alter membrane characteristics in Thermoplasma acidophilium and in specific preparations of synthetic lipids (23,24). Alterations in lipid packing order and fluidity are known to play an important role in growth behavior and differentiation of many cell types (25). Measurements of fluorescence anisotropy for any given cell type are highly reproducable. Furthermore, perterbation of membrane fluidity normally results in activation of homeostatic mechanisms which return the membrane to the original fluid state. Alterations in structure, composition

or dynamic membrane properties which are maintained by the presence of toxic aluminum ions would be expected to influence cellular transport processes and hence cellular metabolism. Experimental and clinical evidence exists suggesting a role for aluminum in microcytic anemia (26,27). It is likely that aluminum plays a role in such induced anemias by altering membrane motional characteristics and disturbing normal transport essential for cellular metabolism. Further, aluminum has been shown to alter phosphoinositide metabolism and transport of glucose and cholinergic amines in rat brain microsomes (28).

Motional constraints placed on the DPH molecule arise when toxic aluminum ions bind to the membrane. Aluminum binding has been suggested to displace membrane associated ions of physiological significance (18). The large charge to radius ratio of aluminum and hence its ability to strongly polarize its immediate environment could be expected to play an important role in the membrane effects observed in these experiments. It seems reasonable to speculate that the polar head regions of membrane lipids are attractive ligands for the reactive aluminum ion. Presumably, aluminum cross-linking of these polar ligands at the membrane surface is translated deeply into the membrane resulting in alterations in the environment of the fluorescent probe employed in these experiments.

Although aluminum intoxication has been shown to exhibit a

multitude of effects both in vivo and in vitro, no single mechanism stands out as a primary lesion in the pathology of aluminum. Interaction of aluminum ions with the cellular membrane would certainly be an initial stage in any form of aluminum toxicity. Subsequent alteration of membrane structure and dynamics arising from such an interaction could be expected to have a great number of deleterious effects on transport processes and cellular metabolism.

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ALUMINUM-INDUCED CHANGES IN CALMODULIN

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INTRODUCTION

Calcium serves as a second messenger in bioregulation via various intracellular calcium trigger proteins such as calmodulin. As a result of a large variety of external stimuli, intracellular calcium transients are generated which can be interpreted as a signal. Within the lifetime of these transients, calcium ions are bound (signal input) to trigger proteins which undergo conformational changes. These changes play a key role in signal amplification and transmission (output) from the trigger protein to respective target enzymes and structural elements [1]. In view of this central reliance of cellular control on calcium ions and a few trigger proteins, severe repercussions on biochemical and physiological processes can be expected when the coupling between signal input and output is interrupted.

In the following we report on the interaction of toxic aluminum ions with calmodulin which can severely antagonize the biochemical trigger activity of the regulatory protein. Aluminum toxicity is a serious global problem for crop productivity [2,3,4] and for human health [5,6]. As a fringe benefit of investigating aluminum-induced, pathological alterations of calmodulin and associated activities, deeper insight is gained into the protein's physiological mode of action as signal transmitter. To rationalize pathophysiological observations in light of calcium's role as intracellular signal and the impact of aluminum ions on calmodulin, the hypothesis has been formulated that calmodulin is a key lesion in the broadly defined syndrome of aluminum toxicity [7,8].

PHYSICO-CHEMICAL PROPERTIES OF ALUMINUM AND CALCIUM

Basic parameters governing metal cation binding to organic ligands (Table I) lie at the heart of understanding the interaction of aluminum ions with calmodulin. Key factors determining the formation of metal complexes with ligands are the ionic charge, ionic size, and the coordination geometry. The aluminum ion has a high ratio of charge/radius, e/r, which profoundly influences the extent of ionic solvation. At acidic pH values, the aluminum ion is hexahydrated forming an octahedral arrangement of water molecules. As a result of the strongly polarized O-H bond, these solvated aluminum ions hydrolyze in a

Table 1. Physico-Chemical Properties of Aluminum and Calcium Ions [9,13,14].

	Ca^{2+}	Mg^{2+}	Al^{3+}
unhydrated radius (nm)	0.099-0.118	0.066	0.051
hydrated radius (nm)	0.412	0.428	0.475
e/r*	20.2	30.3	58.8
coordination number	8 >7 >6 >9	6	6
hydration enthalpy kcal/mol of ion)	-399	-477	-1141
hydrolysis constant	12	11	5
k_{ex} (sec^{-1})#	10^8	10^5	0.13

*e/r represents charge in elementary units per unhydrated radius in nm.

#k_{ex} is the approximate rate constant for water exchange at 25°C.

pH -dependent manner [9]. At physiological pH, and at micromolar aluminum concentration, singly and doubly charged monomeric species, e.g., $[Al(OH)(H_2O)_5]^{2+}$, are present in aqueous solution. Calcium ions, on the other hand, are not hydrolyzed, or only slightly, in the neutral pH region. Ca^{2+} ions can have variable coordination numbers [10,11], and thus the associated, variable ionic radii, afford the calcium cation significant flexibility in selecting a stereochemically fitting binding site, usually oxygen atoms, compared with the fixed coordination geometry of the aluminum and magnesium ion. An additional distinguishing feature for metal affinities, K_d, to ligands, e.g., a protein, is determined by the release rate of water molecules from the primary solvation shell of the respective cation, k_{on}, and by the dissociation rate of the cation from the protein, k_{off}, where $k_{off} = K_d \cdot k_{on}$ [9,12]. On-rates for calcium binding to calcium-modulating proteins like calmodulin are about 10^8 $M^{-1} \cdot sec^{-1}$, yielding k_{off} values ranging from 100 to about 10 sec^{-1}. Calcium-calmodulin is therefore a suitable trigger for biochemical reactions occurring within seconds to milliseconds. As a result of the higher e/r ratio of the aluminum ion, a much slower rate of water release is to be expected. The magnesium ion has a rate k_{on} of about $10^5 M^{-1} sec^{-1}$, a value close to that measured for the rate of water exchange from the hydrated Mg^{2+} cation [9]. The rate of metal dissociation and the accompanying conformational change of the protein are slow.

ALUMINUM IONS INDUCE HELIX-COIL TRANSITIONS IN CALMODULIN

Upon application of stoichiometric quantities of aluminum ions to micromolar concentrations of calmodulin the negative ellipticity of the protein, measured at 222 nm, decreases with increasing aluminum concentrations (Fig. 1). This indicates that aluminum addition to calmodulin, at a molar ratio of 4:1, decreases the helix content by about 20-30 percent [7], whereas calcium addition to the apoprotein promotes helix formation [1]. Major structural changes in the protein are induced upon addition of the first two aluminum ions. The steepness of the response, as characterized by a Hill coefficient of about 1.55, apparently reflects positive cooperativity and is typical of small

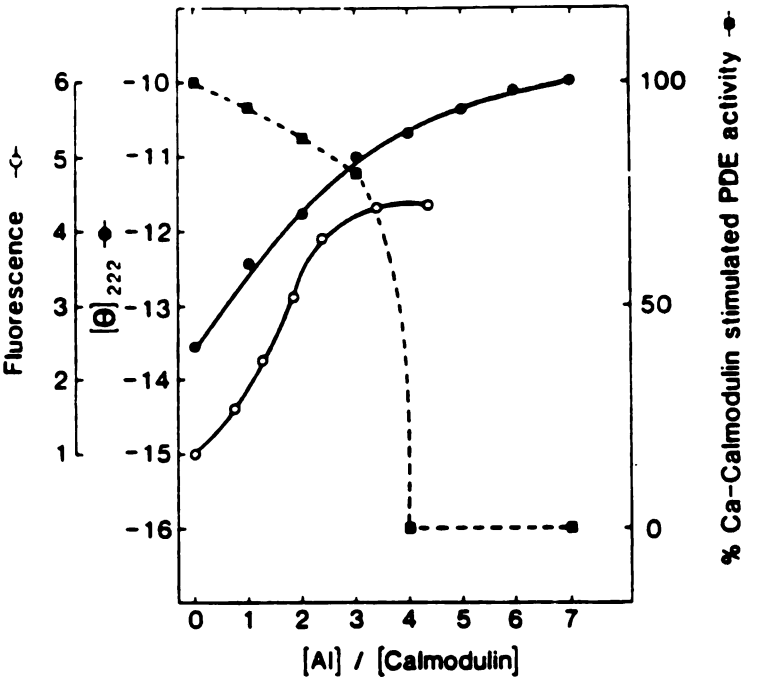

Fig. 1. Mean Residue Ellipticity, $[\theta]_{222}$, ANS Fluorescence Intensity, and 3':5 -Cyclic Nucleotide Phosphodiesterase Activity upon addition of Aluminum Ions to Bovine Calmodulin. For optical studies, the calmodulin concentration was 10 μM, pH 6.5, 23°C, in 10 mM MOPS buffer, 2 μM ANS. ANS excitation at 360 nm, emission recorded at 490 nm. The enzyme assay was performed with 5 μM calmodulin, 10 mM Tris-HCl, pH 6.5, 37°C; hydrolysis of cyclic GMP was assayed.

compact proteins [15]. Aluminum dissociation constants, K_d, have been obtained varying between 0.1 and 0.4 μM [7,16], similar to those of "high-affinity" calcium binding [17]. Stoichiometric binding of aluminum ions to calmodulin takes place irrespective of the presence or absence of saturating calcium concentrations. The aluminum-induced structural changes of calmodulin do not appear to result simply from a displacement of calcium ions from their specific sites, as demonstrated by measuring the protein's calcium content with atomic absorption techniques. Rather, application of aluminum ions to calmodulin, at a molar ratio of up to 8:1, causes binding of additional calcium ions, presumably at non-specific sites [18]. It is also doubtful whether aluminum ions can replace calcium ions at all, considering the differences in charge, etc (Table I).

The aluminum-induced breakage of hydrogen bonds is thus associated with a helix-coil transition as opposed to the calcium-promoted coil-helix formation. As opposed to calcium binding [19], thermodynamic studies demonstrate that binding of the first aluminum ion to the protein is an enthalpy-driven process, while binding of three additional aluminum ions seems to be entropy-driven when coordinated water molecules are released from the coordination sphere upon formation of the aluminum-calmodulin complex [16].

Concomitant with the aluminum-induced helix-coil transition, the hydrophobic surface exposure of the protein is enhanced [7], relative to that observed in the presence of stoichiometric quantities of calcium ions [1]. This was demonstrated in studies by measuring the fluorescence emission of a probe, 8-anilino-1-naphthalene sulfonate (ANS), probably adsorbed near region III [20], probably at a molar ratio around 1:1 [7]. These interpretations are consistent with fluorescence studies using internal tyrosyl residues [17], and NMR data [21,22].

INHIBITION OF CALMODULIN-DEPENDENT ENZYMES BY ALUMINUM IONS

Aluminum-induced helix-coil transitions are accompanied by functional changes of calmodulin which is an activator for a variety of enzymes. For example, at a molar ratio of 4:1 for [aluminum]/[calmodulin], the activity of the calcium- and calmodulin-dependent 3':5'-cyclic nucleotide phosphodiesterase was completely blocked as determined from the hydrolysis of cyclic GMP. Control experiments showed that aluminum interfered with calmodulin rather than with the enzymatic protein [7]. Similarly, micromolar concentrations of aluminum ions interfered with calcium- and calmodulin-dependent Ca^{2+}/Mg^{2+}-ATPase activity which plays a role in the maintenance of the transmembrane potential of plasma membrane enriched vesicles isolated from barley roots. The aluminum-induced reduction of membrane potential build-up, in the presence of ATP, was assayed with fluorescent voltage-sensitive probes [23]. Considering the importance of the transmembrane potential and cellular processes, aluminum-triggered imbalances of the potential are thus expected to have severe repercussions on cell growth and root elongation, consistent with pathophysiological observations [2].

DYNAMICS OF CALMODULIN INTERACTION WITH TARGET PROTEINS

Perturbations of this regulatory protein, e.g., by aluminum ions, are expected to lead to changes in the finely tuned association between transmitter protein and its target, thereby eliciting perhaps nonphysiological responses. Since little information is available with respect to molecular mechanisms responsible for calmodulin/target interaction, melittin was selected as target protein for aluminum-altered calmodulin. Melittin is an amphiphilic polypeptide (M=2846) composed of 26 amino acids, it harbours a single tryptophanyl residue, has a calmodulin affinity in the nanomolar range, and competes with target enzymes for calcium-calmodulin. Therefore, this small protein has been proposed as a model system for the study of calmodulin/target interactions [24].

To derive molecular parameters pertinent for the interaction of melittin with calmodulin, circular dichroism and time-resolved fluorescence anisotropy studies were performed. Melittin's single tryptophanyl residue was used as an intrinsic probe monitoring the microenvironment at the melittin/calmodulin interface, in the presence or absence of stoichiometric amounts of aluminum ions. Compared with calcium-calmodulin, the motional freedom of the tryptophanyl residue is somewhat restricted when melittin is associated with aluminum-altered calmodulin (4:1), probably as a result of hydrogen bonding. Furthermore, in the presence of aluminum ions, the increase of melittin's helix content is reduced, presumably as a result of weaker hydrophobic interactions between the two proteins. A more polar environment of the tryptophanyl residue is also indicated by a shorter fluorescence lifetime

Table 2. Fluorescence Lifetime, t, Bimolecular Quenching Constant for Acrylamide, k_q,and Rotational Correlation Time, ϕ, of Melittin's Tryptophanyl Residue upon Association with Calmodulin (10 μM), in the Presence and Absence of Aluminum. Aluminum was present at a molar ratio of 4:1 for [aluminum]/[protein]. All experiments were performed in the presence of calcium at a molar ratio of 8:1 for [calcium]/[protein] at 25°C. The buffer consisted of 45 mM MOPS-KOH, pH 6.5, 0.2 N KCl. The optical studies were carried out on a SLM spectrofluorimeter, model 4800.

Melittin-Calmodulin	t nsec	ϕ_A nsec	$k_q 10^{-9}$ $M^{-1}sec^{-1}$
Aluminum absent	2.84	8.42	1.20
Aluminum present	2.39	3.54	1.62

(2.39 nsec vs.2.84 nsec when aluminum is absent) and by a smaller blue-shift in the tryptophanyl absorption maximum, when melittin is associated with aluminum-altered calmodulin. Aluminum-induced breakage of hydrogen bonds on calmodulin necessarily leads to a rearrangement of water molecules. Aluminum-altered calmodulin has therefore a solvation structure different from that of the compact calcium-calmodulin. The existence of a more open structure for aluminum-altered calmodulin is consistent with our findings that quenching molecules have better access to melittin's tryptophanyl residue when melittin associates with aluminum-altered calmodulin as compared with calmodulin in the absence of aluminum. Control experiments indicated that application of an eight old excess of aluminum ions over melittin alone did not change the protein's circular dichroism spectrum.

CONCLUSIONS

The results presented in this study demonstrate that application of micromolar concentrations of aluminum ions leads to profound alterations of calmodulin's structure, in the presence or absence of saturating calcium concentrations. Possible binding sites of aluminum ions are carboxyl groups at the surface of the acidic protein. One such binding site may be at, or in close proximity of, region III, because of the enhanced tyrosyl emission, possibly from tyr 99, and because this region has been implicated as a possible binding region of the amphiphilic melittin [24]. The hydrophobic domain of region III is probably also involved in the specific interaction of calcium- calmodulin with certain target enzymes [24].

Upon interaction of aluminum ions with calmodulin, whether in the presence or absence of calcium ions, the conformational states and charge of the regulatory protein are altered. This can be inferred from the aluminum-induced changes in helix content, the reduced electrophoretic mobility of aluminum-altered calmodulin in gels, the enhanced mobility of spin probes attached to the protein [8,16], and the increased hydrophobic surface exposure, compared with corresponding properties of calcium-calmodulin. Aluminum-induced overall structural changes of the protein necessarily impact the local conformation at the specific calcium binding loops. Although calcium ions are rather flexible as to the respective coordination geometry, the ease of departure of the calcium cation, i.e., its rehydration, may be significantly altered. If the

off-rate becomes too slow, say slower than about 1 sec^{-1}, calmodulin's trigger capability for signal transmission in biochemical reactions is probably impaired. To illustrate this point, upon addition of trifluoperazine to calmodulin, the off-rate for Ca^{2+} from calmodulin is reduced by about tenfold [25], compared with the off-rate in the absence of the inhibitor.

Since structural fluctuations appear to play a crucial role in protein dynamics and catalysis [26,27], aluminum binding to calmodulin perturbs the dynamic equilibrium existing between the protein's energy states. This type of equilibrium is partially governed by the presence of solvation water, whose amount and type of association with respective amino acid residues are interrelated with the protein's conformation, e.g., that established in the presence of aluminum ions. As a result, certain states of the aluminum-altered solvated protein are no more (or less) accessible which, in turn, prevents the proper fit between trigger molecule and target protein. Moreover, the respective "non-physiological" energy state is virtually frozen, because the off-rate for aluminum ions is extremely small (Table I), qualitatively speaking of the order of $k_{off} < 10^{-7}$ sec^{-1}, assuming a binding constant of about 10^7 [7]. Consequently, the dynamic interaction between such a calmodulin and its target is greatly diminished. Long retention times of aluminum bound to carboxylate ligands have also been found in NMR experiments [28].

As a result of aluminum binding, melittin's association with calmodulin, probably in region III [24], seems to be diminished compared with calcium-calmodulin in the absence of aluminum. As to the size of the interface, it was hypothesized that a minimum structural requirement of melittin binding to calmodulin is a basic, amphiphilic helix of about 1.5 nm length [29]. A weaker association is reflected in less helix formation of the peptide, because the interface site becomes more polar and displayed altered dynamic changes upon binding of aluminum to the calmodulin. It is interesting to point out that region III forms a compact structure only in the presence of Ca^{2+} and shows positive cooperativity with the adjacent region IV , while the domain pairs (I and II) and (III and IV) show negative cooperativity [30]. We lack information to ask whether aluminum binding perturbs the respective cooperative interaction among the four domains of the regulatory protein?

CHELATORS PROTECT CALMODULIN FROM ALUMINUM INJURY

Since multifunctional calmodulin has been highly conserved during evolution, the hypothesis was put forward [31], that protective mechanisms should exist that protect the pivotal protein from aluminum injury. This notion is supported by observations that certain aluminum-tolerant plant species are rich in aluminum-chelating compounds such as organic acids [2]. Indeed, our circular dichroism and ANS fluorescence experiments demonstrate that citric acid, at a molar excess of [citrate]/[aluminum], can protect calmodulin from aluminum injury, if the metal is presented to the protein in stoichiometric amounts. These findings are consistent with results from ^{27}Al-NMR experiments indicating that citrate forms a stable complex with aluminum between pH 5 and 8.0 [28]. Control experiments showed that the presence of a tenfold excess of citrate over calmodulin (10 µM) did not interfere with calcium binding to the protein (4:1), again in accord with known stability constants for citrate/calcium binding.

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APPENDIX B

DYNAMIC PROPERTIES OF CALMODULIN IN RESPONSE TO ALUMINUM

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KEY WORDS: CALMODULIN, FLUORESCENCE ENERGY TRANSFER, ALUMINUM

Aluminum, the most abundant metal in the earth's crust, has been implicated as early as 1918 as a cause of root-growth retardation in crop plants grown on acidic soil (1). Today, aluminum toxicity is recognized as a serious global problem for crop productivity because vast areas of the world suffer from soil acidity which mobilizes soil aluminum and thus can be taken up by plants (2,3). Besides natural soil acidity, anthropogenic perturbation of hydro- geochemical processes has further contributed to the acidification of terrestrial and aquatic regions previously not suffering from aluminum toxicity (4). For example, so-called acid rain has substantially increased over wide parts of the eastern United States, Canada, and western Europe (5). As to human health, clinical and experimental findings suggest a link between aluminum uptake and a variety of disorders in man, e.g., osteomalacia-type diseases and dialysis encephalopathy (6).

Little is known about molecular mechanisms regarding entry of aluminum species into cells via the plasma membrane, about primary targets for these species, or about aluminum-induced cellular lesions. Deeper insight into cellular and molecular processes involving aluminum ions is crucial for the biotechnological development of plant resistance to aluminum toxicity. Despite this dearth of information, cellular membranes and calcium-regulated biochemical processes appear to emerge as potential targets for toxic aluminum ions.

Concerning aluminum interactions with membranes, a body of evidence suggests pronounced aluminum-induced phase changes in the plasma membrane of thermophilic Archaebacteria (7) and in plasma membrane-enriched microsomal fractions isolated from maize roots (8). Furthermore, micromolar aluminum concentrations induced membrane rigidification and membrane fusion in unilamellar lipid vesicles containing phosphatidylserine (9).

Besides interfering with the plasma membrane, physiological studies indicated that aluminum ions effect changes in calcium regulation (2,6). Therefore, a potential target for aluminum interaction might be calmodulin, a pivotal , calcium-dependent regulatory protein (M=17,000). This acidic protein has been highly conserved during evolution of eukaryotic cells where it partici- pates in a multitude of cellular processes (10). Given the significance of calmodulin in integrating calcium-dependent processes, aluminum-induced changes of the protein's structure would therefore have severe repercussions on cellular functions. Indeed, recent investigations indicate that binding of aluminum ions to bovine brain calmodulin results in pronounced structural and dynamic changes which, in turn, lead to the inhibition of crucial calcium- and calmodulin-dependent enzymes (3,11,12).

As to aluminum-induced structural changes, the metal binds stoichiometrically and cooperatively to the protein,

as determined by fluorescence and circular dichroism studies. Binding of aluminum occurs irrespective of the presence or absence of calcium ions (11,13). When bound to the protein, aluminum ions trigger a helix-coil transition whereby the helix content decreases by about 30 percent. It is noteworthy, that addition of calcium ions promotes helix formation (10), with aluminum absent. Atomic absorption experiments indicated that the application of excess citrate to an aluminum-calmodulin (3:1) complex resulted in a time-dependent, quantitative removal of the metal from the protein (14). As judged by spectroscopic techniques, this citrate- mediated protein restoration is somewhat incomplete in terms of helical content. The incomplete rearrangement of calmodulin structure is probably related to aluminum-induced changes in solvation. The rearranged water molecules may compete for hydrogen bonding sites on calmodulin when the protein refolds as a result of citrate-mediated aluminum removal.

Concomitant with aluminum-induced changes in helix content, the hydrophobic surface exposure of calmodulin is enhanced (3), relative to the area observed in the presence of stoichiometric quantities of calcium ions (10). This hydrophobic region or its immediate vicinity probably play a key role in the interaction of calmodulin with specific partner proteins, e.g., an ATPase (10). Aluminum-related changes at the interface between calmodulin and a partner

protein were illustrated in experiments with melittin (15), a small protein which has a high affinity with calcium-calmodulin (16). As monitored at the single tryptophanyl residue of melittin, the fluorophore's average microenvironment is modified such that its apparent lifetime is shortened when aluminum is present. Moreover, the interface between the two proteins seems to become more polar when aluminum is bound to calmodulin. In the presence of aluminum, calmodulin's solvation structure is possibly altered, which may be unfavorable for a proper fit between calmodulin and the target protein.

We present in this article further results on aluminum-related structural and dynamic changes monitored by a fluorescent dye attached to the single cysteinyl residue of spinach calmodulin. By measuring molecular parameters like fluorescence anisotropy, motional parameters were derived which indicate that aluminum altered calmodulin differs from native calcium-calmodulin.

Experimental

Mastoparan-X and mastoparan were purchased from Peninsula Laboratories (Belmont, CA). N-iodoacetyl-N'-(5-sulfo-1-naphthyl) ethylene-diamine (1,5-I-AEDANS) was obtained from Sigma Chemical Company (St. Louis, MO). Affinity

chromatography material, phenothiazine Affi-gel, and the protein assay kit were purchased from Bio-Rad Laboratories (Richmond, CA). All other chemicals used were of the highest quality available.

Calmodulin was isolated from fresh spinach using methods previously described (14,17). Spinach calmodulin obtained in this manner was found to be free of tryptophan as judged by UV absorbance and lack of emission intensity at 340nm following excitation with 295nm light.

To attach the fluorescence probe, 1,5-I-AEDANS, to spinach calmodulin, the protein was first dissolved in Tris buffer (10 mM, pH 7.0) at a concentration of about 0.2 mg/ml buffer, then a thiol-reducing agent, dithiothreitol, was added to the solution to a final concentration of 0.2 mM. The reaction was allowed to proceed at room temperature for 18 hours. The reacted sample was then extensively dialysed against the same Tris buffer to remove excess thiol-reducing agent. The fluorescence label, 1,5-I-AEDANS, was added and reacted with the sample as described (17). The reaction mixture was finally dialyzed against Mops buffer (10 mM at pH 6.5 and 0.1 M KCl). The molar ratio of AEDANS bound per protein was found to be 0.95 to 1, as determined by dye absorbance
(ϵ = 6 100 cm^{2} $^{-1}$ mM at 337 nm) (19) and Bradford assay (20), respectively.

Fluorescence experiments (anisotropy, lifetime) were perfomed on an SLM spectrofluorimeter, model 4800 (Urbana, IL), as described previously (15). Briefly, the steady-state polarization anisotropy, r, of the fluorescence is

$$r = (I_{//} - I_{\perp})/(I_{//} + 2I_{\perp}) \quad (1)$$

where $I_{//}$ and $I_{\perp}$ refer to the intensity of the polarized emission measured parallel and perpendicular with respect to excitation. The emission anisotropy is related to the lifetime of the fluorescence, τ, and rotational correlation time, ϕ, according the the Perrin equation (21):

$$r = r_o(1 + \tau/\phi)^{-1} \quad (2)$$

where r_o refers to the anisotropy in the absence of molecular motion. Fluorescence lifetimes were calculated from phase shift and demodulation data (21). It was assumed that the fluorescence decay process could be analyzed in terms of a biphasic decay, viz., a fast and short-lived decay, both exponential in time and independent from each other.

Fluorescence energy transfer was evaluated between the single tryptophanyl residue on mastoparan-X (M = 1,557) and the fluorescence label, AEDANS, residing at the single cysteinyl residue, Cys-26 (22), of spinach calmodulin. As

opposed to mastoparan-X, mastoparan (M = 1,479) does not harbor any tryptophanyl residue. The energy transfer efficiency was measured by the donor quenching method (23), i.e.,

$$E = 1 - Q_{DA}/Q_D \qquad (3)$$

where E stands for the energy transfer efficiency. Q_{DA} and Q_D refer to the fluorescence quantum efficiency of the donor, in the presence or in the absence of the acceptor. The ratio Q_{DA}/Q_D can be evaluated from corresponding fluorescence intensity measurements, f_{DA} and f_D, according to

$$Q_{DA}/Q_D = [f_{DA}/f_D]_{av} \times [D]_D/[D]_{DA}. \qquad (4)$$

Here, both fluorescence intensities, f_{DA} and f_D. are measured upon excitation of the donor at 299 nm. The quantities, f_{DA} and f_D, are the fluorescence intensities of the donor measured at the same wavelength free from the acceptor emission. Subsequently the intensity ratios were averaged. $[D]_D$ is the concentration of donor molecules in the absence of any acceptor, whereas $[D]_{DA}$ is that in the presence of both acceptor and donor molecules.

The distance, R, from the donor to the acceptor was

calculated from the following equation,

$$R = R_o\ (1/E\ -1)^{1/6} \qquad (5)$$

where R_o is the Förster critical distance, evaluated from

$$R_o^6 = (8.79 \times 10^{-5}) \times k^2 \times n^{-4} \times D \times DA \qquad (6)$$

in which J_{DA} is the spectral overlap integral defined as

$$J_{DA} = \{\sum_{\lambda} f_D(\lambda) \cdot \varepsilon_A(\lambda) \cdot \lambda^4 \cdot \Delta\lambda\} / \{\sum_{\lambda} f_D(\lambda) \cdot \Delta\lambda\} \qquad (7)$$

where ε_A is the molar extinction coefficient of the acceptor in $M^{-1}cm^{-1}$; λ is the wavelength in nm. k^2 is the orientation factor and a value of 2/3 was used in these experiments. Usage of this value is justified if donor and acceptor molecules randomize prior to energy transfer (24). Since the aromatic ring systems of the donor (tryptophan) (21) and the acceptor (AEDANS) have two linear transition moments each, the assumption of random orientation appears to be reasonable.

In summary, four kinds of samples have to be prepared for these energy transfer studies: (a) calmodulin plus mastoparan (neither donor nor acceptor present), (b) calmodulin plus mastoparan-X, i.e. (D); (c) AEDANS-labelled calmodulin plus mastoparan, i.e. (A); and (d)

AEDANS-labelled calmodulin plus mastoparan-X, i.e., (DA).

Results

The emission of the AEDANS-spinach calmodulin conjugate reaches a maximum at 490 nm and is considerably blue-shifted from the emission maximum of 520nm reported for the aqueous free probe. An emission maximum observed at 490 nm corresponds to that reported for AEDANS dissolved in 60% ethanol/water (19). When covalently bound to spinach calmodulin, the probe apparently resides in an environment less accessible to water. Increased rigidity or further water removal in the probe's microenvironment may be responsible for the blue shift. The fluorescence spectra of dye-labelled spinach calmodulin, measured in the presence or absence of micromolar aluminum or calcium concentrations, virtually coincide.

Table I lists the values of various spectroscopic parameters of the dye-labelled calmodulin in the presence and absence of calcium and aluminum. The average fluorescence lifetime of the probe remains relatively constant through serial calcium titration and upon addition of aluminum up to a molar ratio of 3 aluminum ions per protein. However, the rotational correlation time decreases with increasing calcium concentration, consistent with reports that the protein adopts a more compact conformation

Table I. Fluorescence properties of AEDANS-labelled spinach calmodulin at various cation concentrations

sample	r	$a^*_{1,2}$	$\tau^*_{1,2}$ (ns)	τ_{av} (ns)	ϕ_{av} (ns)
AEDANS-calmodulin	0.0579	0.14 0.86	0.88 9.86	9.7	15.0
AEDANS-calmodulin +2 Ca^{2+}	0.0524	0.12 0.88	0.57 10.44	10.4	12.6
AEDANS-calmodulin +5 Ca^{2+}	0.0528	0.14 0.86	0.55 9.71	9.6	11.7
AEDANS-calmodulin +5 Ca^{2+} + 3 Al	0,0575	0.13 0.87	0.08 9.94	9.9	14.9

* a and τ represent the preexponential factors and lifetimes analyzed on the basis of a biphasic decay.
r is the fluorescence anisotropy measured by T-format method as indicated in legend to Figure 2, except at room temperature.
τ_{av} are average lifetimes calculated from the relations

$$\tau_{av} = (\alpha_1\tau_1^2 + \alpha_2\tau_2^2)/(\alpha_1\tau_1 + \alpha_2\tau_2)$$

ϕ_{av} values are the rotational correlation times calculated from Equation 2.

upon calcium binding (25). Upon further addition of aluminum, the rotational correlation time significantly increases relative to the time determined in the absence of aluminum. The correlation times were calculated from Equation 2 using an r_o value of 0.0910 $\pm$ 0.0035. This value was obtained from a Perrin plot by extrapolation with a correlation coefficient of 0.988.

Our fluorescence lifetime data can be interpreted in terms of a biphasic fluorescence decay. The lifetime of the long-lived component roughly coincides with that measured for free AEDANS in water (19). Since our instrument does not permit measurements of time-resolved anisotropy decays, an average correlation time had to be calculated (Table I).

To assess the impact of aluminum on the interaction between calmodulin and its target proteins, radiationless energy transfer experiments were performed between the single tryptophanyl residue on the partner peptide, mastoparan-X, and the AEDANS label on Cys-26 on spinach calmodulin. These small peptides are known to bind calmodulin with high affinity in a calcium-dependent manner (16). The fluorescence emission band of tryptophan shows excellent spectral overlap with the acceptor's absorption spectrum, AEDANS- conjugated cysteine (Figure 1), and therefore, energy transfer from tryptophan to the acceptor can be expected. By integrating in steps of 2 nm, the value of the overlap integral, J_{DA}, was calculated according to

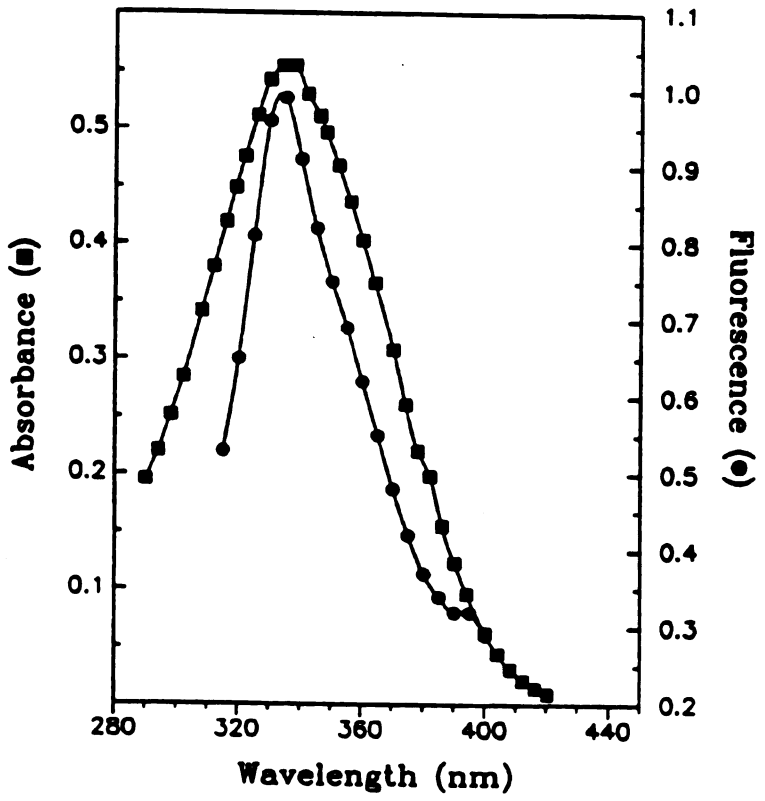

Figure 1.-The spectral overlap of the fluorescence emission (●) of the single tryptophanyl residue in mastoparan-X (donor), associated with spinach calmodulin, and the absorption (■) of AEDANS- conjugated cysteine (acceptor). The samples were dissolved in 4-morpholinopropane sulfonic acid (Mops) buffer (10 mM, pH 6.5, 0.1 M KCl and 0.15 mM $CaCl_2$). The concentration of mastoparan-X was 20 μM, while that of the spinach calmodulin was 30 μM. The concentration of AEDANS- conjugated cysteine was 9.3 μM. Tryptophan was excited at 299 nm, and both experiments were conducted at room temperature.

Equation 7. Assuming a donor quantum yield, Q_D, of 0.155 (26), a refractive index of n = 1.334, corresponding to that of the 0.1 M KCl solution at room temperature (27), and an orientational factor of $k^2 = 2/3$, the Förster critical distance, R_o, can be determined (Table II). The Förster critical distance , R_o, is in accord with that reported for the radiationless energy transfer from from Trp-19 of melittin to AEDANS-labelled troponin C at Cys-98, viz., R_o = 23.6 A° (28). Since the spectral characteristics of donor emission and acceptor absorption did not change when aluminum ions were added to the calcium-containing sample, the overlap integral and therefore R_o remained the same (Table II). The efficiency of energy transfer between donor and acceptor was higher when aluminum was present in the sample, resulting in a distance of 29.5 A°. In the absence of aluminum, this distance was 30.3 A° (Table II).

To further evaluate aluminum-related dynamic changes of spinach calmodulin, the influence of temperature on the fluorescence anisotropy of AEDANS-conjugated spinach calmodulin was determined (Figure 2). In the presence of saturating calcium concentrations, there appear to be discontinuities in slope which depend upon the presence or absence of aluminum ions. At the lower temperature, changes in slope are observed at 25°C, with aluminum present, relative to a value of 22°C in the presence of calcium

Table 2. Energy transfer characteristics of AEDANS-labelled spinach calmodulin in the absence and in the presence of aluminum ions

sample	energy transfer efficiency (E)	overlap integral (J_{DA})	R_o (A°)	R (A°)
calmodulin + 5 Ca^{2+}	0.194 ± 0.014	6.50×10^{13}	23.9	30.3
calmodulin + 5 Ca^{2+} + 3 Al	0.219 ± 0.009	6.50×10^{13}	23.9	29.4

R_o is the Förster critical distance. R is the the distance between donor and acceptor molecule. The experiments were performed at room temperature.

Table 3. Molecular parameters of AEDANS-labelled spinach calmodulin

sample	temperature (C°)	anisotropy	τ_{av} (ns)	ϕ_{av} (ns)
calmodulin	18.2	0.0571 ± 0.0003	17.3	29.1
+ 5 Ca^{2+}	28.5	0.0486 ± 0.0003	8.5	9.7
calmodulin	18.2	0.0616 ± 0.0003	12.0	25.1
+ 5 Ca^{2+} + 3 Al	28.5	0.0525 ± 0.0003	9.2	12.5

Symbols equivalent to those in Table I.

only. Corresponding changes are observed at 37°C and at 33°C, respectively. These kinds of changes in slope probably reflect temperature-induced structural changes of the protein, rather than changes attributable to the dye itself. This conclusion is supported by observations of similar thermal changes in calmodulin as studied by microcalorimetry and intrinsic tyrosine fluorescence (29). Moreover, temperature-induced structural changes of calmodulin are also reflected in molecular parameters representative for those of the protein (Table III). The molecular parameters were evaluated at two temperatures, viz., one below, the other one above the temperature indicative of protein thermal changes. At 18.2°C, the rotational correlation time, 29.1 ns, measured for AEDANS-labelled spinach calmodulin in the presence ofcalcium, is in general accord with values measured at different wavelengths for bovine calmodulin (30). At 18.2°C, the rotational correlation time is faster, when aluminum is present, compared with that in the absence of aluminum. At 28.5°C, however, this time pattern is reversed (Table III).

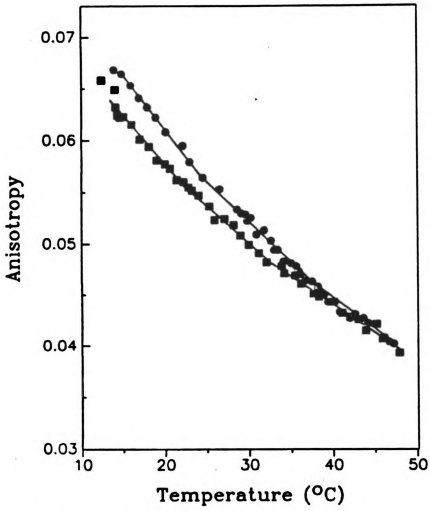

Figure 2.-Fluorescence anisotropy values of AEDANS-labelled spinach calmodulin versus temperature, in the presence and in the absence of aluminum ions. The experiments were performed according to the T-format method on a spectrofluorimeter, model SLM 4800. Two identical Corning cut-off filters, CS-72, were placed in the emission beams. The SLM instrument was interfaced with a microcomputer, HP-85, for data analysis. Both samples were excited at 344 nm. Calmodulin concentration was 10 uM. (■), [calmodulin]:[Ca^{2+}] =1:5; (●), [calmodulin]:[Ca^{2+}]:[aluminum] = 1:5:3.

Discussion

X-ray diffraction methods revealed that the equilibrium average structure of calmodulin consists of a dumbbell-shaped protein in which the two lobes are interconnected by an eight-turn α-helix (31). The C-terminal lobe of calmodulin harbors the two high- affinity calcium binding sites (10). Like other globular proteins, calmodulin is also a dynamic system which comprises a multitude of conformational substates, easily accessible by rapid structural fluctuations (32,33). These conformational substates are in part dependent on the viscosity of the solvent (34). Our understanding of the molecular dynamics of proteins is in its infancy, however, evidence is accumulating that at least some of these conforma- tional substates are crucial for protein function (33,35). Indeed, we have demonstrated in this article that application of aluminum ions perturbs the conformation of calmodulin existing in the presence of calcium ions. As a consequence, Al-changed calmodulin lost its capacity to stimulate calcium- and calmodulin-dependent enzymatic proteins (11,12,14).

As monitored by the dye attached to the single cysteinyl residue 26 of spinach calmodulin, application of aluminum ions to calcium-calmodulin (5:1) results in changes of motional properties different from those with aluminum

absent. These types of aluminum-related changes in calmodulin are detectable although the extent of hydrophobicity of the probe's microenvironment appears to resemble that observed in the absence of calcium ions. When measured at room temperature, the rotational correlation time of spinach calmodulin apparently becomes faster as the calcium concentration increases up to a molar ratio of 5 for $[Ca^{2+}]$/ [calmodulin]. Compared with apocalmodulin, calcium-calmodulin appears to be more compact. These observations are in accord with findings on bovine calmodulin (30). An apparent calcium-induced compaction might be also achieved when apocalmodulin, which has an ellipsoidal shape, becomes slightly bent as a result of calcium addition. Bending of calmodulin is possible because of the protein's inherent flexibility (36).

As the sample temperature decreases, the rotational correlation time increases, viz., up to 29.1 ns at 18°C for AEDANS-labelled spinach calmodulin in the presence of saturating calcium concentrations. Aluminum-altered calmodulin qualitatively exhibits the same trend as to the temperature-dependence of the rotational correlation time. On the other hand, our data on the influence of temperature on the fluorescence anisotropy suggest that application of aluminum to spinach calmodulin produces a calmodulin structure distinctly different from that in the absence of aluminum, especially at the lower temperatures measured.

Since aluminum application breaks hydrogen bonds in calmodulin (11,15), transitions between certain energy minima of the protein are facilitated which -- in the absence of aluminum -- would be less accessible. In the plot of the fluorescence anisotropy versus temperature, discontinuities in slope may reflect rearrangements in the solid- and liquid-like microdomain structure of the protein (35). This kind of intramolecular reorganization in aluminum- altered calmodulin is probably responsible for the protein's inability of stimulating enzymes such as cyclic nucleotide phosphodiesterase (3).

The experiments on energy transfer from mastoparan-X's tryptophanyl residue to the AEDANS label at Cys-26 of spinach calmodulin show a higher transfer efficiency in the case of aluminum-altered protein. The small amphiphilic peptide, mastoparan-X, appears to bind to calmodulin's region III, when calcium is present (37). Therefore a distance of about 30 A^{o} between donor and acceptor appears to be reasonable, considering the location of AEDANS at Cys-26 in region I.

In summary, upon application of aluminum ions to calcium- calmodulin, dynamic changes occur which apprently make it impossible for the protein to fulfill its role in stimulating calmodulin-dependent enzymes.

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APPENDIX C

pH-RELATED CHANGES IN MAIZE ROOT PLASMA MEMBRANES

CHARLES SUHAYDA, CHRISTOPHER WEIS, and ALFRED HAUG

KEY WORDS: ALUMINUM, MEMBRANE, pH, ZEA MAYS L.

INTRODUCTION

The detrimental effects of soil acidity upon plant growth usually include factors such as hydrogen ion concentration, calcium and phosphorus deficiencies, and excess of aluminum[1]. As to direct effects of pH on plant growth, the ambient hydrogen ion activity was found to play a significant role in the growth of leguminous plants[2]. Particular attention has been directed to the role of the hydrogen ion concentration in mobilizing aluminum from soil[1,3]. The strong effect of pH on aluminum solubility is rather pronounced in the range from pH 4.0 to 6.0[4]. In part facilitated by the presence of bicarbonate and/or chelation with organic ligands, aluminum may be transported from soil to the aqueous environment where the metal seems to be toxic to fish at concentrations above 4-8 μmol/L[5]. The complex interplay between pH and aluminum is further confounded by the fact that polymeric aluminum cations provide some solution buffering[5]. Moreover, by comparing the differential tolerance to aluminum in certain wheat cultivars, stress tolerance seems to be related to the cultivar's ability of maintaining a high pH value, thus diminishing aluminum solubility[6].

Changes in environmental pH also influence the balance of electrical charges on cellular membranes, the maintenance of electrical gradients, the permeability, and possibly cytosolic pH regulation and metabolic activities.

Understanding plant growth on acidic soils therefore requires a thorough knowledge of the physico-chemical status of the soil and of interactions of hydrogen ions (and aluminum) with cells. In this report we therefore describe experiments on the effect of pH on the pH gradient and the physical state of the plasma membrane of vesicles derived from maize roots.

MATERIAL AND METHODS

Sealed membrane vesicles were isolated from maize roots[7] of the cultivar MO17xWF9. For measurements of fluorescence polarization anisotropy, 40 µL of membrane dispersions were introduced into a quartz cuvette containing 3 ml of buffer. The buffer contained 1 mM 1,3-bis[tris (hydroxy-methyl)-methylamino]- propane (BTP) adjusted to the appropriate pH value by addition of 2-[N-morpholino]ethane sulfonic acid (MES). A 1 mg/ml stock solution of a fluorescence probe, trimethylammonium diphenyl hexatriene (TMA-DPH)[8], was prepared in dimethyl formamide. 0.5 µL of TMA-DPH solution were added to the membrane suspension in the quartz cuvette. Using a Neslab refrigerated circulating water bath, model RTE-8, the sample temperature was maintained at 20° or 30°C, respectively. The sample was excited at 360 nm and fluorescence emission was recorded through Corning filters No. 3060 on an SLM spectrofluorimeter, model 4800 (Urbana, IL). Since

scattering can cause depolarization, the optical density at 360 nm was checked at each pH value tested. The sample temperature was monitored ($\pm$ 0.1°C) by an Omega digital thermometer, model 410B-T.

Optical measurements were performed to determine the vesicle pH gradient. Proton transport in sealed membrane vesicles was assayed by the quenching of quinacrine fluorescence, at room temperature[7]. The dye was excited at 430 nm and fluorescence emission was recorded at 500 nm. The fluorescence dye apparently accumulates inside the membrane vesicles as the internal pH is lowered. Dye accumulation leads to fluorescence quenching which serves to monitor proton pumping activity.

RESULTS AND DISCUSSION

In plasma membrane-enriched vesicles, isolated from maize roots, pronounced changes in fluorescence anisotropy occur between the pH values 5.5 and 6.5, at the temperatures investigated (Table 1). These changes are monitored by a fluorescent probe, TMA-DPH, a cationic derivative of the fluorescence probe, DPH. The charged trimethyl ammonium group of TMA-DPH is probably anchored at the polar heads of the membrane phospholipids, while the apolar moiety, DPH, resides in the membrane's hydrophobic region. A sensitive parameter for pH-induced membrane changes is the fluorescence anisotropy indicative of viscosity changes in

the probe's microenvironment; the larger the microviscosity, the larger the steady-state anisotropy value[9].

TABLE 1
pH Dependence of Fluorescence Anisotropy of Plasma Membrane Vesicles Labelled with TMA-DPH.

pH	anisotropy	
	20°C	30°C
6.5	0.2909 ± 0.0004	0.2739 ± 0.0008
5.5	0.3068 ± 0.0008	0.2896 ± 0.0005

At physiological temperatures, the anisotropy increases upon acidification of the membrane's suspending medium. Proton-induced changes in intermolecular interactions and decreased electrostatic repulsions between membrane constituents apparently lead to a tighter packing of proteins and lipids. These kinds of membrane changes are similar to those observed in plasma membrane-enriched microsome fractions isolated from barley roots where a reduction of lipid head group mobility was associated with decreased membrane-bound Ca^{2+} -and Mg^{2+}-dependent ATPase activity[10].

This pH-induced membrane reorganization is probably related, at least in part, to nutrient uptake of the plant

root in an acidified environment. In rye grass, for example, cation absorption is sharply reduced below pH 5[11]. Moreover, the membrane's high permeability of rice roots under acidic stress could be reduced by addition of high calcium concentrations[12], which are known to modulate membrane properties[13].

Proton pumping activity in inside-out vesicle preparations was activated upon addition of Mg^{2+} and ATP. The ATPase activity appears to be associated with the plasma membrane, rather than the tonoplast, because addition of 200 μM sodium orthovanadate, a known inhibitor of plasma membrane bound ATPase activity[14], to 300 μg membrane protein inhibited the pumping activity by 65% at pH 6.5. Moreover, the Mg^{2+}-ATPase activity was unaffected by oligomycin, an inhibitor of mitochondrial ATPase[14]. These data suggest that the membrane fraction employed in these studies was predominantly of plasma membrane origin.

As assayed by quinacrine fluorescence quenching (Fig. 1), proton pumping activity reached a steady state level at about 750 sec after ATP application. At this time there was sufficient ATP left to sustain the steady state level of proton pumping as defined by quinacrine quenching. This steady state level probably reflects a state of balance between ATP-dependent proton pumping and proton leakage across the vesicle membrane[7].

The pH gradient across the vesicular membrane is strongly pH dependent. Taking the intensity of quinacrine fluorescence at 750 sec relative to that observed initially as an indicator for proton

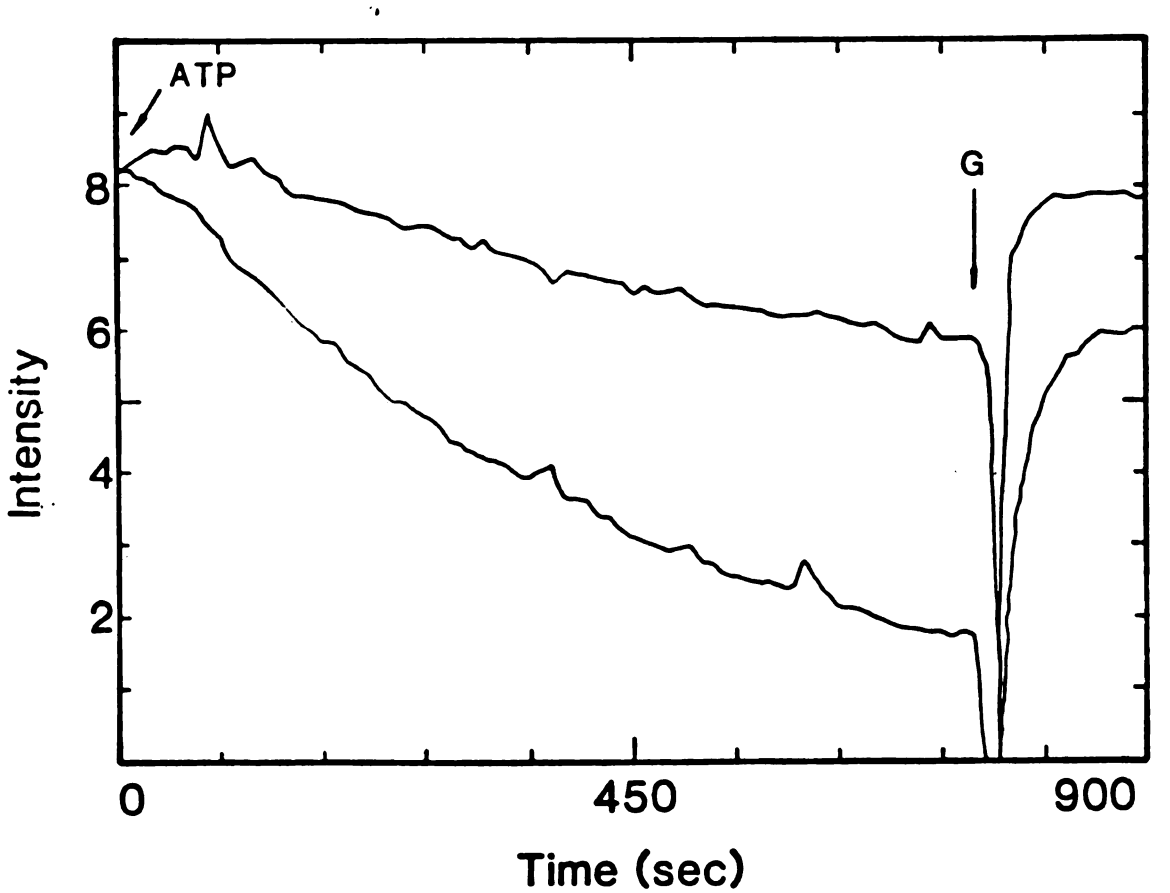

FIG.1 - Measurement of Proton Pumping Activity in Maize Root Vesicles by Quinacrine Fluorescence Quenching. The assay mixture contained 300 μg membrane protein, 250 mM sorbitol, 3 mM $MgSO_4$, 10 μM quinacrine, and 3 mM ATP (BTP salt), and 25 mM BTP buffer adjusted to pH 6.5, or pH 5.5, with MES. Application (G) of gramicidin (5 μM final concentration) discharged the proton gradient.

pumping efficacy, the pumping activity at pH 6.5 is 3.5-fold that measured at pH 5.5 (Fig. 1). A diminished pumping activity at the lower pH value may be a consequence of pH-related changes in membrane organization. Indeed, a decrease in lipid acyl chain fluidity in the "boundary lipid" region surrounding the Mg^{2+}-ATPase has been found to result in inhibition of Ca^{2+}- and Mg^{2}- dependent ATPase activity in barley root plasma membranes[10]. In membrane vesicles derived from maize roots the proton electrochemical gradient could be partially discharged upon application of 5 μM gramicidin to the assay mixture. Control experiments indicated that pumping activity did not occur in the absence of ATP.

Since a variety of cellular processes may respond to pH-induced changes in plasma membrane structure and proton gradients[15], it would be important to understand the interrelationship between the external hydrogen cation concentration and biochemical activities of plants.

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