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DYNAMIC MOTIONS OF CALMODULIN IN RESPONSE TO ALUMINUM BINDING: AN IMPLICATION FOR ALUMINUM TOXICITY

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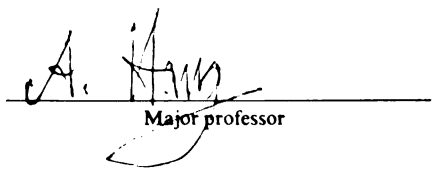

Major professor

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DYNAMIC MOTIONS OF CALMODULIN IN RESPONSE TO ALUMINUM BINDING: AN IMPLICATION FOR ALUMINUM TOXICITY

By

Shixing Yuan

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ABSTRACT

DYNAMIC MOTIONS OF CALMODULIN
IN RESPONSE TO ALUMINUM BINDING:
AN IMPLICATION FOR ALUMINUM TOXICITY

By

Shixing Yuan

The dynamic motions of spinach calmodulin in the nanosecond range can be studied through a strategically anchored probe, e.g., a fluorescence probe, bimane, or, a EPR probe, proxyl, attached at cysteinyl-26 residue. With Ca^{2+} ions present, fluorescence studies indicated that the rotational motions of calmodulin is faster relative to those of apocalmodulin. This may result from compactness of calmodulin conformation in the presence of calcium, which implies that when Ca^{2+} ions bind to calmodulin, local conformational changes can trigger a largescale molecular conformational rearrangement. This explanation also matches the studies in binding of mastoparan to calmodulin molecule. Fluorescence studies showed when mastoparan binds to calomdulin, the whole mastoparan-calmodulin complex rotates slower, i.e., the rotational correlation time becomes longer because the total volumn of the complex becomes larger than calmodulin molecule itself. On the other hand, mastoparan binding also triggers largescale

conformational rearrangement on calmodulin molecule, which can be monitored through the microenvironmental changes around the EPR probe. When aluminum ions were added to calmodulin solution, in the presence of saturation of Ca^{2+}, motional changes of calmodulin different from those in the absence of aluminum ions were observed. Fluorescence energy transfer studies showed that the energy transfer efficiency from the single tryptophanyl residue on mastoparan-X to the probe attached at the cysteinyl site is different in the presence of aluminum from that in the absence of aluminum. This suggests that aluminum ions may interfere the interactions between calmodulin and mastoparan. Also, the Y-function studies showed the temperature dependent interchange of two substates of calmodulin molecule is totally different in the presence of aluminum from that in the absence of aluminum. With aluminum prsent, the interchange needs (or releases) more energy. This means that the conformation of calmodulin becomes less flexible in reponse to temperature changes in the environment in the presence of aluminum. We concluded that these conformational changes triggered by aluminum ions may in part constitute aluminum toxicity mechanisms in the eukaryotic world.

Dedicated to my dear brother, Shizhong Yuan.

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Chapter I

LITERATURE REVIEW

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Introduction:

Aluminum is the most abundant metal in the crust of the earth. Until recently, aluminum was believed to have no biological function. Recent studies indicate, however, that Al^{3+} can activate G proteins which are involved in signal transduction across the plasma membrane (1). Furthermore, an increasing number of toxic effects has been shown to be associated with aluminum ions. Aluminum becomes toxic when it is mobilized under acidic conditions and enters into the biosphere (2, 3, 4, 5). This mobilization was suggested happening at the root-soil interface (6). Since around 40% of the arable land of the world and perhaps up to 70% of potentially usable land are acidic and the plants in those areas are subject to aluminum toxicity, this toxicity represents a formidable barrier to successful food and biomass production around the world (7). Plants grown on acidic subsoil are highly susceptible to drought and have less access to subsoil nutrients since aluminum can limit the plant root development and penetration into subsoils and thus increasing the drought susceptibility of the plants and limiting their access to mineral nutrients (8). The mobile aluminum ions exert their toxic effects on the fine roots of afflicted plants and result in significant losses in root structure and the eventual dieback of the aerial portions of these plants (9). Also when higher plants were exposed to $AlCl_3$ at 800 or 1200 µmol/l for a long time the contents of both Mg and Ca in roots and needles of the seedlings could be reduced (10). Some aluminum-tolerant plants, e.g., aluminum-tolerant Triticum aestivum, can overcome this toxic effect by

preventing aluminum ions from entering symplasm or by some internal detoxification mechanisms (6). When red spruce are exposed to aluminum ions and simulated acid precipitation, a symptom of calcium deficiency will take place (11), resulting in the cessation of plant growth. It is believed that aluminum can inhibit calcium uptake and translocation (12). In addition, the deposition of acid rain in the northeast U.S. and Canada has led to the transport of labile monomeric aluminum (compared with the complexes existing at basic pH) ions in the soil and enhanced the concentration of this metal ion in lakes and surface waters (13, 14). This implied that under acidified conditions, aluminum toxicity could be the primary cause of death in animals such as teleosts (15, 16, 17).

Until recently, aluminum toxic effects upon human body has not been described. Now, it is commonly accepted that some dialysis diseases, e.g., dialysis encephalopathy and dialysis osteomalacia, are caused by aluminum ions (18, 19, 20). Also, accumulation of aluminum has been related to cellular aging and death (21). Some other diseases like senile dementia of the Alzheimer's type (22, 23), amyotrophic lateral sclerosis (24), and idiopathic Parkinsonism (21) are suggested to be related to aluminum toxicity.

Since the acidification of the environment and its implied aluminum toxicity problem are drawing more and more attention, it will be important for us to get a better understanding of the mechanisms of how aluminum affects living systems, especially the Ca^{2+}-related regulatory processes in vivo. Since calmodulin is a key factor in these processes, and a possible target of aluminum toxicity (3, 21, 25.). It

is my aim in this dissertation to study the biophysical aspects of aluminum toxicity at the molecular level, i.e., aluminum ions affecting conformation of calmodulin molecule and the protein's interaction with the targets.

General Properties of Aluminum Ions

Estimates indicate that the average concentration of aluminum in the crust of the earth is about 81,000 ppm, and the total amount of aluminum accounts for about 7.5% the weight of the crust (26). In nature, aluminum exists only in the oxidation state (III). Since the ion is in a strong charged state (usually as Al^{3+}), the ionic radius for aluminum is small, only 0.51 A^{o}. With this high ratio of charges over radius (58.8 for Al^{3+}, 30.3 for Mg^{2+} and 20.2 for Ca^{2+}), the nonhydrated aluminum ion has a strong polarizing effect on adjacent atoms. At highly acidic pH, the aluminum ion is trivalent and hexahydrated, $Al(H_2O)_6^{3+}$, forming an octahedral configuration with water molecules, while at neutral pH, the formation of hydroxo-complex polymers, e.g., $AlO_4Al_{12}(H_2O)_{12}(OH)_{24}^{7+}$, will be promoted as hydrolysis happens. Acidification leads to a decrease of polymers while increasing the concentrations of free single, double and triple charged monomers, $Al(H_2O)_6^{3+}$, $Al(H_2O)_5(OH)^{2+}$ and $Al(H_2O)_4(OH)_2^{+}$ (27). In complexes, Al^{3+} is generally bound to oxygen. Consequently, acidic oxygens of membrane lipids, carboxyl groups of proteins and chromatin material, e.g., phosphate groups of DNA, make ideal target ligands for cationic

aluminum. Aluminum can also bind nitrogen, but with lower affinity. The ligand exchange (e.g., change from Al-O to Al-N) is slow partly due to a fairly marked covalent contribution to the Al-O and Al-N bonds. Slowness of the ligand exchange is also due to other things, e.g., the exchange generally occurs by a dissociative mechanism (28), and the complexes are often chelates by six ligands. For an aluminum ion, the coordination number is strictly 6, while the formation of dissociative intermediate needs the number higher than 6. This makes the formation unlikely. The slowness explains why the toxicity and other effects of Al^{3+} are easily underestimated in short-term experiments.

Aluminum ion is the major component of a large number of minerals. At neutral pH, the solubility of aluminum minerals is extremely low, but increases as pH decreases. Acid rain and usage of acidifying fertilizers acidify the environment and increase the concentration of soluble Al^{3+} in soil and in lakes and rivers. It is not the low pH but rather the subsequent increase of dissolved Al^{3+} in the water that causes gill damage and death among young fish. Also, the elevated concentration of Al^{3+} in lakes have caused serious and sometimes lethal toxic effects on birds living in the immediate surroundings (29).

Al^{3+} dissolved in soil is one of the foremost growth limiting factors in many parts of the world (30). This probably results also from the ready ability of Al^{3+} to bind root and to prevent the uptake of inorganic phosphates. Al^{3+} seems also to specifically inhibit root growth of plants (31, 32, 33).

Aluminum content in human body was estimated between 30 - 50 mg

(34, 35). About half of this is in the skeleton and a quarter in the lungs. Basically, all the aluminum comes from food intake except that in the lungs. The absorption of Al^{3+} varies when suitable complexing substances are also present, for example, the presence of phosphate can prevent Al^{3+} uptake just as Al^{3+} hydroxide prevents phosphate uptake (36).

Aluminum Interaction with Proteins

Aluminum has been shown to inhibit hexokinase (37 - 42) which results from the formation of a complex of ATP-Al^{3+}. This complex acts as a strong competitive inhibitor with respect to ATP-Mg^{2+}. In this way, Al^{3+} can inhibit ATP-dependent enzymes, such as, Na^{+}-K^{+} ATPase (43). Al can also inhibit some other enzymes such as: adenylate cyclase (44, 45), 3', 5'-cylic nucleotide phosphodiesterase (46) serum cholinesterase (47), alkaline phophatase (48, 49), acetylcholinesterase (50, 51), catechol-O-methyl-transferase (52), and ferroxidase (53).

Among the important regulatory proteins interacting with Al^{3+}, calmodulin is one (46, 54) whose activities can be greatly affected by aluminum binding. The study of the interaction between calmodulin molecule and aluminum ions will be the main theme of this thesis.

Characteristics of Calmodulin

Calmodulin is a small (M_r = 16,000 - 17,000), acidic protein (pI = 4.1 - 4.2) existing in all species of the eukaryotic world.

Calmodulin molecule is quite conserved during evolution (Fig. 1.1) (25). According to reports, calmodulin isolated from the protozoan Tetrahymena has eleven substitutions and one deletion when compared to bovine brain calmodulin (55). This homology demonstrates that all the calmodulins have similar conformations and functions. So far, all calmodulin molecules are involved in regulating calcium related processes in vivo. Like all members of troponin C superfamily, a calmodulin molecule has the characterized helix-loop-helix, the so-called EF-hand structures (Fig 1.2) for Ca^{2+} binding (56). Among its four Ca^{2+} binding sits, the two near the C-terminal can be defined as high affinity binding sites ($K_d = 10^{-7}M$), while the other two are low affinity ones ($K_d = 10^{-6}M$) (57). The whole molecular structure of calmodulin is like a dumbbell, two lobes connected with an 8-turn alpha-helix rod (58, 59) (Fig. 1.3). Each lobe contains two Ca^{2+} binding sites (58, 59). In solution, a calmodulin molecule is not in the rigid dumbbell conformation, instead, the rod connecting these two lobes is fairly flexible (60).

In the absence of calcium binding to calmodulin, the molecule is in the inactive state. But when calcium binds, calmodulin becomes activated and can interact with numerous regulatory enzymes, which, in turn, can regulate different processes in the cell (61) (Fig. 1.4). The activated form of calmodulin can either be Ca^{2+}_2-calmodulin or Ca^{2+}_{3-4}-calmodulin (62, 63, 64, 65). Calmodulin exhibits hydrophobic interactions with its target enzymes or peptides. Amphiphilicity plays an important role in this interaction. When calmodulin binds Ca^{2+}, more hydrophobic surface is exposed. This

Fig. 1.1. Amino acid sequence of calmodulin. The basic sequence shown here is that from bovine brain. Wherever there is a substitution in any different species, it is shown by an arrow. Otherwise, the sequence is the same.

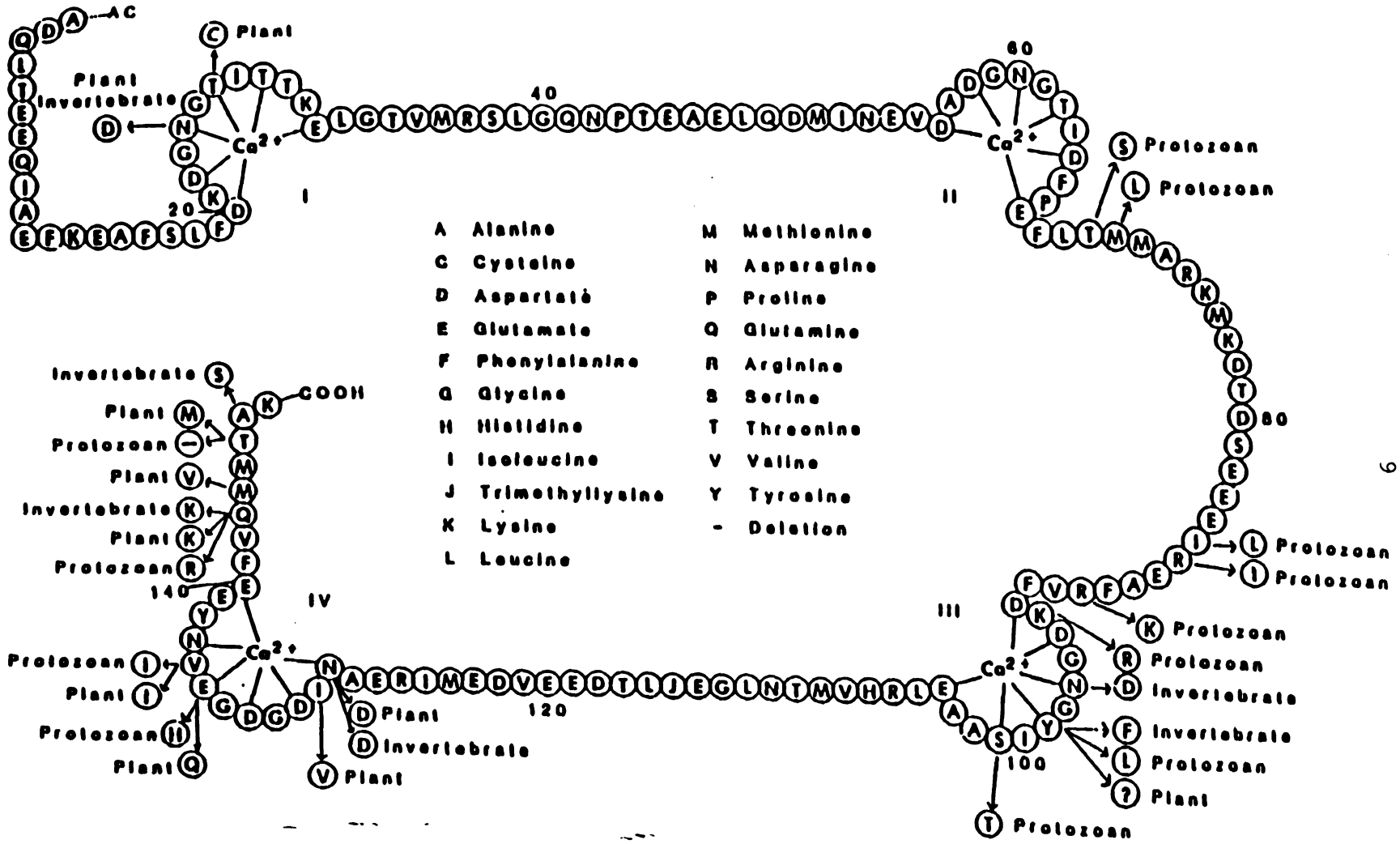
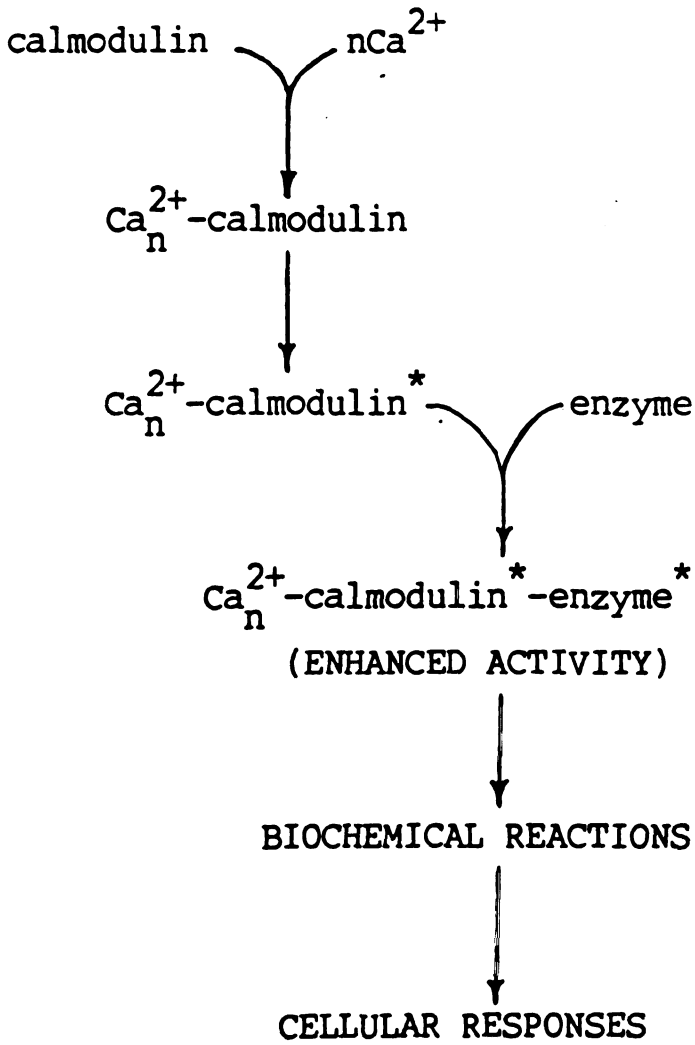

A Alanine
C Cysteine
D Aspartate
E Glutamate
F Phenylalanine
G Glycine
H Histidine
I Isoleucine
J Trimethyllysine
K Lysine
L Leucine
M Methionine
N Asparagine
P Proline
Q Glutamine
R Arginine
S Serine
T Threonine
V Valine
Y Tyrosine
- Deletion
I
II
III
IV
20
40
60
80
100
120
140
Ca2+
COOH
Plant
Invertebrate
Protozoan

Fig. 1.2. EF-hand structure of Ca^{2+}-related superfamily molecules. o stands for a liganded Ca^{2+}. Each Ca^{2+} here is liganded by six oxygen atoms. An EF-hand is characterized by a stretch of helix-loop-helix structure.

Fig. 1.3. Stereo drawing of the alpha-C of calmodulin. O stands for the liganded Ca^{2+}.

Fig. 1.4. Calmodulin-related regulatory pathways. * stands for the activated form of calmodulin or an specific enzyme. Here, the enzyme is any of the calmodulin-dependent enzymes, e.g., adenylate cyclase, guanylate cyclase, phosphodiesterase, phospholipase A_2, NAD kinase, phosphorylase kinase, myosin light chain kinase and glycogen synthase kinase. The calmodulin-dependent processes include cellular motility, cytoskeletal function, glycogen metabolism, cyclic nucleotide metabolism, calcium metabolism and photosythesis.


calmodulin
nCa2+
Can2+-calmodulin
Can2+-calmodulin*
enzyme
Can2+-calmodulin*-enzyme*
(ENHANCED ACTIVITY)
BIOCHEMICAL REACTIONS
CELLULAR RESPONSES

exposed surface could then be essential for the target binding. Several neural toxic peptides, such as mellitin and mastoparan, are able to inhibit calmodulin activities by specifically interacting with this exposed surface (66).

Aluminum Effects on Calmodulin

Aluminum has already been shown to have effects on calcium regulation. The accumulation of aluminum in plants can inhibit calcium transportation across the membrane and induce calcium deficiency syndrome. Increase of calcium concentration can alleviate or eliminate these effects. Since calmodulin is a key factor in the Ca^{2+}-related regulatory processes in vivo, the complex formed between calmodulin and aluminum ions may be a key lesion in the syndrome of aluminum toxicity (46).

The application of aluminum ions to calmodulin causes pronounced structural changes in the latter molecule (46). The metal binds stoichiometrically and cooperatively to calmodulin. Binding of aluminum to calmodulin at a molar ratio of 2:1 leads to a reduction in the alpha helix of the protein as monitored by ultra-violet circular dichroism (46), and an increase in the hydrophobic surface area as indicated by the fluorescent hydrophobic surface probe 1-anilino-8-naphthalene-sulfonic acid (ANS) (46). These changes could result in a spatial geometry rearrangement between calmodulin and its peptide ligand, mastoparan. Also, binding of aluminum to calmodulin results in a concomitant decrease in the ability of calmodulin to activate

3', 5'-cyclic nucleotide phosphodiesterase (46). The presence or absence of calcium does not interfere with aluminum binding (46, 67). Taken together, aluminum binding to calmodulin introduces a perturbation into the conformation of calmodulin and this perturbation, in turn, may signal a toxicological effect of this metal. Partial reversal of the toxic effects of aluminum ions on calmodulin by citrate has been documented (68). Citrate can 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 (69). Estimates from spectroscopic data indicated that the binding affinity for the first mole of aluminum ions bound to the protein is about one order of magnitude stronger than that of calcium.

Aluminum appears to interact directly with calmodulin (46). At a molar ratio of 3:1 of [Al]:[calmodulin], the calmodulin-stimulated membrane-bound ATPase activity in barley root plasma membrane vesicles was severely inhibited. The metal-induced changes in calmodulin structure were reflected in a reduced formation of a transmembrane potential when assayed with a voltage-sensitive fluorescent probe (70).

Application of aluminum to calmodulin may induce breakage of calmodulin's hydrogen bonds which are associated with enthalpic effects such as changes in steric and electrostatic repulsions and can results in dramatic conformational changes (71).

The observed changes in aluminum-induced hydrophobic surface may be accompanied by changes in calmodulin solvation structure since solvation effects contribute significantly to protein stability (72,

73). Al-calmodulin appears to be different in protein flexibility and in its ability to interact with target proteins, compared with corresponding characteristics of the calcium-calmodulin structure. These changes in calmodulin may explain why the aluminum-calmodulin complex lost part of its regulatory character.

LIST OF REFERENCES

1. Gilman, A.G., (1987) G proteins: transducers of receptor-generated signals. Ann. Rev. Biochem. 56: 615-649.

2. Haug, A., (1984) Molecular aspects of aluminum toxicity. CRC Crit. Rev. Plant Sci., 1(4): 345-373.

3. Baes, C.F., and Mesmer, R.E., (1976) The Hydrolysis of Cations. John Wiley and Sons, New York.

4. Burgess, J., (1978) Metal Ions in Solution. John Wiley and Sons, New York.

5. Fratiello, A., (1972) Nuclear magnetic resonance cation solvation studies, in: Inorganic Reaction Mechanisms Part II, (Edwards, J.O. ed.), Interscience, New York.

6. Taylor, G.J., (1988) The physiology of aluminum tolerance in higher plants. Commun. in Soil Sci. Plant Anal., 19(7-12): 1179-1194.

7. Osmond, C.B., Bjorkman, O., and Anderson, D.J., (1980) in: Physiological Processes in Plant Ecology. Springer-Verlag, Berlin. pp. 41-66.

8. Foy, C.D., Chaney, R.L., and White, M.C., (1978) The physiology of metal toxicity in plants. Ann. Rev. Plant Physiol. 29: 511-566.

9. Ulrich, B., Mayer, R., and Khanna, P.D., (1980) Chemical changes due to acid precipitation in loess-derived soil in central Europe. Soil Sci. 130: 193-199.

10. Godblod, D.L., Fritz, E., and Huttermann, A., (1988) Aluminum toxicity and forest decline. Proc. Natl. Acad. Sci. (U.S.) 85: 3888-3892.

11. Shortle, W.C., and Smith, K.T., (1988) Aluminum-induced calcium deficiency syndrome in declining red spruce. Science 240: 1017-1018.

12. Ownby, J.D., and Dees, L., (1985) Growth and mineral nutrient status in peanut and sorghum in response to acid precipitation and aluminum. New Phytol. 101: 325-332.

13. Driscoll, C.T., and Newton, R.M., (1985) Chemical characteristics of Adirondack lakes - Individual lakes respond differently to acid deposition. Environ. Sci. Technol. 19(11): 1018-1024.

14. David, M.D., and Driscoll, C.T. (1984) Aluminum speciation and equilibria in soil solutions of a haplorthod in the Adirondack

Mountains (New York, U.S.A.). Geoderma 33: 297-318.

15. Cronan, C.S., and Schofield, C.L., (1979) Aluminum leaching response to acid precipitation: effects on high elevation watersheds in northeast. Science 204: 304-306.

16. Brown, D.J.A., (1983) Effect of calcium and aluminum concentrations on the survival of Brown Trout at low pH. Bull. Environ. Contam. Tocicol. 30: 582-587.

17. Alfrey, A.C., LeGendre, G.R., and Daehney, W.D., (1976) The dialysis encephalopathy syndrome. Possible aluminum intoxication. N. Eng. J. Med. 294: 184-188.

18. Ellis, H.A., McCarthy, J.H., and Herrington, J., (1979) Bone aluminum in haemodialysed patients and in rats infected with aluminum chloride: relationship to impaired bone mineralization. J. Clin. Pathol. 32: 832-844.

19. Driscoll, C.T., Jr., Bader, J.P., Biosogni, J.J., Jr., and Schofield, C.L., (1980) Effect of aluminum speciation on fish in dilute acidified waters. Nature 284: 161-164.

20. Burnatowska-Hledin, M., Kaiser, L., and Mayer, G.H., (1983) Aluminum, parathyroid hormone, and osteomalacia. in: Special Topics in Endocrinology and Metabolism, 2. 201-226.

21. Ganrot, P.O., (1986) Metabolism and possible health effects of aluminum. Environ. Health Perspect. 65: 363-441.

22. Crapper, D.R., Karlik, S., and DeBoni, U., (1978) Aluminum and other metals in senile (Alzheimer) dementia. in: Alzheimer's Disease: Senile Dementia and Related Disorders, (Aging, Vol. 7) (Katzman, R., Terry, R.D., and K.L. Bick, eds.), Raven Press, New York. pp. 471-495.

23. Trapp, G.A., Miner, G.D., Zimmerman, R.L., Mastri, A.R., and Heston, L.L., (1978) Aluminum levels in brain in Alzheimer's disease. Biol. Psychiat. 13: 709-718.

24. Garruto, R.M., Fukatsu, R., Yanagihara, R.C., Gajdusek, D.C., Hook, G., and Fiori, C.E., (1984) Imaging of calcium and aluminum in neurofibrillary tangles-bearing neurons in Parkinsonism-dementia of Guam. Proc. Natl. Acad. Sci. (U.S.) 81: 1875-1879.

25. Klee, C.B., and Vanaman, T.G., (1982) Calmodulin. Adv. Protein Chem. 35: 213-321.

26. Lepp, N.W., (1981) Chapter 2, Other trace metals, aluminum. in: Effect of Heavy Metal Pollution on Plants, Vol. 1., Applied Science, London. pp. 214-218.

27. Smith, R.W., (1971) Relations among equilibrium and nonequilibrium aqueous species of aluminum hydroxocomplexes. in: Nonequilibrium systems in natural water chemistry (Adv. Chem. Ser. 106) American Chemical Society, Washington D.C., pp. 250-279.

28. Burgess, J., (1978) Chapter 11, Kinetics and mechanisms: solvent exchange. in: Metal Ions in Solution. Ellis Horwood Ltd., Chichester., pp. 310-348.

29. Nyholm, N.E.I., (1981) Evidence of involvement of aluminum in causation of defective formation of eggshells and of impaired breeding in wild passerine birds. Environ. Res., 26: 363-371.

30. Foy, C.D., (1974) Effects of aluminum on plant growth. in: The Plant Root and Its Environment. (Carson, E.W., ed.), Univ. Press of Virginia, Charlottesville., pp. 601-642.

31. Morimura, S., Takahashi, E., and Matsumoto, H., (1978) Association of aluminum with nuclei and inhibition of cell division in onion (Allium cepa) roots. Z. Pflanzenphysiol. 88: 295-401.

32. Clarkson, D.T., (1965) The effect of aluminium and some other trivalent metal cations on cell division in the root apices of Allium cepa. Ann. Bot. N. S., 29: 309-315.

33. Sampson, M., Clarkson, D., and Davies, D.D., (1965) DNA-synthesis in aluminum treated roots of barley. Science 148: 1476-1477.

34. Tipton, I.H., and Cook, M.J., (1963) Trace elements in human tissues. Part II. Adult subjects from the United States., Health Phys. 9: 103-145.

35. Alfrey, A.C., (1984) Aluminum intoxication. N. Eng. J. Med. 310: 1113-1114.

36. Spencer, H., and Lender, M., (1979) Adverse effects of aluminum-containing antacids on mineral metabolism. Gastroenterology 76: 603-606.

37. Trapp, G.A., (1980) Studies of aluminum interaction with enzymes and proteins -- the inhibition of hexokinase. Neurotoxicology 1: 89-100.

38. Harrison, W.H., Codd, E., and Gray, R.M., (1972) Aluminium inhibition of hexokinase. The Lancet 2: 277.

39. Womack, F.C, and Colowick, S.P., (1979) Proton-dependent inhibition of yeast and brain hexokinase by aluminum in ATP preparations. Proc. Natl. Acad. Sci. (U.S.) 76: 5080-5084.

40. Viola, R.E., Morrison, J.F., and Cleland, W.W., (1980) Interaction

of metal (III)-adenosine-5'triphosphate complexes with yeast hexokinase. Biochemistry 19: 3131-3137.

41. Neet, K.E., Furman, T.C., and Hueston, W.J., (1982) Activation of yeast hexokinase by chelators and the enzymic slow transition due to metal-nucleotide interactions. Arch. Biochem. Biophy. 213: 14-25.

42. Lai, J.C.K., and Blass, J.P., (1984) Inhibition of brain glycolysis by aluminum. J. Neurochemistry 42: 438-446.

43. Staurnes, M., Sigholt, T., and Reite, O.B., (1984) Reduced carbonic anhydrase and Na-K-ATPase activity in gills of salmonids exposed to aluminium-containing acid water. Experientia 40: 226-227.

44. Sternweis, P.C., and Gilman, A.G., (1982) Aluminum, a requirement for activation of the regulatory component of adenylate cyclase by fluoride. Proc. Natl. Acad. Sci. (U.S.) 79: 4888-4891.

45. Mansour, J.M., Ehrlich, A., and Mansour, T.E., (1983) The dual effect of aluminum as activator and inhibitor of adenylate cyclase in the liver fluke Fasciola hepatica. Biochem. Biophys. Res. Commun. 112: 911-918.

46. Siegel, N., and Haug, A., (1983) Aluminum interaction with calmodulin. Evidence for altered structure and function from optical and enzymatic studies. Biochim. Biophys. Acta 744: 36-45.

46a. Siegel, N., and Haug, A., (1983) Inorgan, Chim. Acta 79: 230-231.

47. Marquis, J.K., (1983) Aluminum inhibition of human serum cholinesterase. Bull. Environ. Contam. Toxicol. 31: 164-169.

48. Rej, R., and Bretaudiere, J.-P., (1980) Effects of metal ions on the measurement of alkaline phosphatase activity. Clin. Chem. 26: 423-428.

49. Bamberger, C.E., Botbol, J., and Cabrini, R.L., (1968) Inhibition of alkaline phosphatase by beryllium and aluminum. Arch. Biochem. Biophys. 123: 195-200.

50. Patocka, J., (1971) The influence of Al^{3+} on cholinesterase and acetylcholinesterase activity. Acta Biol. Med. Ger. 26: 845-846.

51. Marquis, J.M., and Lerrick, A.J., (1982) Noncompetitive inhibition by aluminum, scandium and yttrium of acetylcholinesterase from Electrophorus electricus. Biochem. Pharmacol. 31: 1437-1440.

52. Mason, L., and Weinkove, C., (1983) Radioenzymatic assay of catecholamines: reversal of aluminium inhibition of enzymatic

O-methylation by desferrioxamine. Ann. Clin. Biochem. 20: 105-111.

53. Huber, C.T., and Frieden, E., (1970) The inhibition of ferroxidase by trivalent and other metal ions. J. Biol. Chem. 245: 3979-3984.

54. Siegel, N., Coughlin, R., and Haug, A., (1983) A thermodynamic and electron paramagnetic resonance study of structural changes in calmodulin induced by aluminum binding. Biochem. Biophys. Res. Commun. 115: 512-517.

55. Yazawa, M., Yagi, K., Toda, H., Kondo, K., Narita, K., Yamazake, R., Sobue, K., Kakiuchi, S., Nagao, S., and Nozawa, Y., (1981) The amino acid sequence of the Tetrahymena calmodulin which specifically interacts with guanylate cyclase. Biochem. Biophys. Res. Commun. 99: 1051-1057.

56. Moews, P.C., and Kretsinger, R.H., (1975) Refinement of the structure of carp muscle calcium-binding parvalbumin by model building and difference Fourier analysis. J. Mol. Biol. 91: 201-228.

57. Haiech, J., Klee, C.B., and Demaille, J.G., (1981) Effects of cations on affinity of calmodulin for calcium: ordered binding of calcium ions allows the specific activation of calmodulin-stimulated enzymes. Biochemistry 20: 3890-3897.

58. Babu, Y.S., Sack, J.S., Greenhough, T.J., Bugg, C.E., Means, A.R., and Cook, W.J., (1985) Three-dimensional structure of calmodulin. Nature (London) 315: 37-40.

59. Seaton, B.A., Head, J.F., Engelman, D.M., and Rocjards. F.M., (1985) Calcium-induced increase in the radius of gyration and maximum dimension of calmodulin measured by small-angle X-ray scattering. Biochemistry 24: 6740-6743.

60. Heidorn, D.B., Torney, D., and Trewhella, J., (1987) Modelling studies of X-ray solution scattering data for calmodulin: a comparison of the X-ray crystal and solution structures. Biophys. J. 51: 453a.

61. Cormier, M.F., (1983) Calmodulin: the regulation of cellular function. in: Calcium in Biology. (Spiro T.G. ed.), John Wiley and Sons, New York, pp. 53-106.

62. Crouch, T.H., Holroyde, M.J., Collins, J.H., Solaro, R.J., and Potter, J.D., (1981) Interaction of calmodulin with skeletal muscle myosin light chain kinase. Biochemistry 20: 6318-6325.

63. Johnson, J.D., Holroyde, M.J., Crouch, T.H., Solaro, R.J., and Potter, J.D., (1981) Fluorescence studies of the interaction myosin light chain kinase. J. Biol. Chem. 256: 12194-12198.

64. Cox, J.A., Malnoe, A., and Stein, E.A., (1981) Regulation of brain cyclic nucleotide phosphodiesterase by calmodulin. J. Biol. Chem. 256: 3218-3222

65. Huang, C.Y., Chau, V., Chock, P.B., Wang, J.H., and Sharma, R.K., (1981) Mechanism of activation of cyclic nucleotide phosphodiesterase: requirement of the binding of four Ca^{2+} to calmodulin for activation. Proc. Natl. Acad. Sci. (U.S.) 78: 871-874.

66. Anderson, S.R., and Malencik, D.A., (1986) Peptides recognizing calmodulin. in: Calcium and Cell Function. (Cheung W.Y. ed.), Academic Press, Inc. Vol 4, pp. 1-42.

67. Siegel, N., and Haug, A., (1983) Aluminum-induced inhibition of calmodulin-regulated phosphodiesterase activity: Enzymatic and optical studies. Inorgan. Chim. Acta 79: 230-231.

68. Suhayda, C.G., and Haug, A., (1985) Citrate chelation as a potential mechanism against aluminum toxicity in cells: the role of calmodulin. Can. J. Biochem. Cell Biol. 63: 1167-1168.

69. Suhayda, C.G., (1986) Organic acids reduce aluminum toxicity in maize root membranes. Doctoral thesis. Chapter VI pp. 52-82.

70. Siegel, N., and Haug, A., (1983) Calmodulin-dependent formation of membrane potential in barley root plasma membrane vesicles: a biochemical model of aluminum toxicity in plants. Physiol. Plant. 59: 285-291.

71. Hartley, F.R., Burgess, C., and Alcock, R.M., (1980) Chapter 14, The interpretation of stability constant data. in: Solution Equilibria. Halsted Press, Chichester, p. 249.

72. Conway, B.E., (1981) Ionic Hydration in Chemistry and Biophysics. Elsevier, Amsterdam.

73. Franks, F., and England, D., (1975) The role of solvent interactions in protein conformation, CRC Crit. Rev. Biochem., 3: 165-219.

CHAPTER II

LIGAND-TRIGGERED CONFORMATIONAL PERTURBATIONS ELICIT CHANGES AT THE SINGLE CYSTEINYL RESIDUE OF SPINACH CALMODULIN

Shixing Yuan and Alfred Haug

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Following application of stoichiometric amounts of Ca^{2+} or mastoparan, a specific ligand peptide, to spinach calmodulin, dynamic changes in the nanosecond range could be monitored at a strategically anchored fluorescence or spin probe. For these studies the single cysteinyl residue 26 of spinach calmodulin was labelled with a thiol-specific proxyl (i.e., 2,2,5,5-tetramethyl-1-pyrrolidinyl-oxyl) spin probe or with a bimane fluorescence probe. With Ca^{2+} and mastoparan present, fluorescence studies (anisotropy, lifetime) indicated that the rotational motion of the mastoparan-calmodulin complex is slower relative to the motion of calmodulin in the absence of mastoparan. The probe's attachment site (residue 26) appears to reside in a fairly polar microenvironment as reported by a series of proxyl spin probes varying in label length. The rotational correlation time of the shortest spin probe markedly changed upon binding of mastoparan to a calmodulin region distant from that of the monitoring spin probe. We interpret these observations as indicating that ligand-triggered conformational perturbations elicit specific responses at the cysteinyl residue 26 of spinach calmodulin.

Introduction

Calmodulin is a representative of Ca^{2+} binding proteins which mediate a multitude of Ca^{2+}-dependent biochemical processes (1). Calmodulin (M_r=15,700 for spinach calmodulin) is an acidic, heat-stable protein consisting of a single polypeptide chain whose primary amino acid residue sequence is highly conserved during evolution of eukaryotes. The protein has a pronounced tendency to bind 4 Ca^{2+} in its helix-loop-helix structures known as EF hands (2) (also see Chapter I). Ca^{2+} binding to calmodulin is sequential since the first two cations apparently bind to the so-called high-affinity binding sites, III and IV (K_d are about 10^{-7}M.) (1), located in the C-terminal half of the dumbbell-shaped protein (3,4). The remaining two Ca^{2+} bind to the low-affinity binding sites, I and II (K_d are about 10^{-6}M), which reside in the N-terminal lobe of the dumbbell. Upon Ca^{2+} binding calmodulin undergoes conformational changes which expose a hydrophobic region serving as an interface for the interaction between calmodulin and its target proteins. Certain pharmacological agents (5) and aluminum ions (6) possess anticalmodulin activity probably by interfering with this interfacial region.

Calmodulin's multifunctional and regulatory properties are linked to the protein's ability to specifically coordinate Ca^{2+}. Questions of interest are directed towards elucidating mechanisms by which conformational changes, triggered locally by Ca^{2+}, in the presence or absence of a target protein, are globally transmitted within the regulatory protein. This transmission of information in the protein

probably depends in part on structural fluctuations and motions (7,8) which occur on a time scale as fast as picoseconds. In such a dynamic system the potential energy surface seems to comprise a multitude of thermally accessible minima in the neighborhood of the native protein structure (9). The functional significance of these molecular motions is illustrated by findings that myoglobin cannot bind oxygen unless the protein structure fluctuates to permit oxygen passage (10).

Examination of the putative Ca^{2+}-related transfer of information requires studies of the relative motion of calmodulin's constituent regions. Therefore we present in this article results concerning structural changes monitored at a defined locus on calmodulin in response to the interaction of the same protein with its specific ligands (Ca^{2+}, mastoparan) which triggered the response in a region distant from and close to that of the monitoring site. For this purpose we selected spinach calmodulin which harbors a single cysteinyl residue at site 26 of the polypeptide chain (11). A thiol-reactive spin probe or a fluorophore was anchored at this site where ligand-related structural modifications of the probe's microenvironment could be monitored in terms of motional characteristics. We were able to demonstrate that ligand-triggered conformational perturbations seem to elicit specific responses at the site of the strategically anchored probe.

Materials and Methods

Chemicals

The spin labels, 3-maleimido-proxyl (designated as spin label I), 3-(male-imidomethyl)-proxyl (II), 3-(2-maleimido-ethyl-carbamoyl)-proxyl (III), 3-(3-maleimidopropylcarbamoyl)-proxyl (IV), and 3-[2-(maleimido-ethoxy)-ethyl carbamoyl]-proxyl (V) were purchased from Aldrich Chemical Company (Milwaukee, WI). Monobromotrimethyl-ammoniobimane was purchased from Calbiochem (San Diego, CA) under the brand name thiolyte MQ. Mastoparan was obtained from Peninsula Laboratories (Belmont, CA). Affinity chromatography material, phenothiazine Affigel, was obtained from Bio-Rad Laboratories (Richmond, CA). DEAE-Sephadex A50 and buffers, Mops and Tris, were purchased from Sigma Chemical Company (St. Louis, MO).

Purification of spinach calmodulin

Calmodulin was isolated from fresh spinach by using slightly modified procedures which have been described (12,13). Simply speaking, 2 kilograms of fresh spinach leaves was chopped, buffer H (50 mM Tris, pH 8.0, containing 1 mM EGTA, 1 mM mercaptoethanol, and 1 mM phenylmethyl-sulfonyl fluoride) 2000 ml was added into it, followed by homogenization. The homogenized solution was filtrated through cheese cloth and $(NH_4)_2SO_4$ was added to the filtrated solution (351g/l solution). Centrifugation of the solution was followed and the pH value

of the supernatant solution was readjusted to 4.0 with 50% H_2SO_4 saturated with $(NH_4)_2SO_4$. The solution was centrifuged again and the pellet was collected and redissolved in buffer B (10 mM Tris, pH 7.5, 1 mM EDTA, 1 mM mercaptoethanol, and 0.2 M NaCl). The pH value was readjusted to 8.0, and extensive dialysis against buffer B was followed. Another centrifugation was performed, the supernatant was collected and applied to a DEAE-Sephades column (2.5 x 40 cm), which was preequilibrated with buffer B. The column was washed with salt gradient (starting buffer was buffer B, and the limiting buffer was buffer B plus 0.3 M more of NaCl.). The eluant from the DEAE-Sephadex column was monitered with UV absorption spectra and the calmodulin containing peak was collected. Extensive dialysis against buffer A (Tris, 10 mM, pH 7.5, 0.3 M NaCl, 1 mM mercaptoethanol, 5 mM $CaCl_2$), then applied to the phenothiazine affinity column (1.5 x 18 cm) equilibrated with buffer A. The column was washed with 100 ml of the same buffer A, then with 100 ml of buffer A containing 0.5 M instead of 0.3 M NaCl. Finally the protein was eluted with another Tris buffer (10 mM, pH 7.5, 0.5 M NaCl, 1 mM mercaptoethanol, 10 mM EGTA). 50 ml of the eluant were collected, into which 10 mM $CaCl_2$ were immediately added, followed by dialysis against 4000 ml of $(NH_4)HCO_3$ solution (10 mM), then by extensive dialysis against double-distilled water. The sample was finally electrodialyzed against water and lyophilized. The purity of the sample was assessed as follows: (a) Spinach calmodulin was tryptophan-free as judged by the ultraviolet spectrum and the absence of fluorescence emission typical of tryptophan (14). (b) The ultraviolet absorption spectrum has a partially resolved vibrational

structure typical of that known for bovine calmodulin [1], and a molar absorption coefficient, $\mathcal{E}_{276}$ = 1500 $M^{-1}cm^{-1}$, estimated to be half that of highly purified bovine calmodulin, because spinach calmodulin harbors only a single tyrosyl residue as opposed to two tyrosyl residues in bovine calmodulin. (c) Analysis of the calmodulin preparation on 15 percent polyacrylamide gels followed by Coomassie blue staining revealed a single band migrating at an apparent molecular weight of about 17,000, this result being consistent when up to 40 ug protein were loaded per gel lane (12). (d) The concentration of spinach calmodulin was measured according to the Bradford procedure (15) with a protein assay kit commercially available from Bio-Rad (Richmond, CA) and using bovine plasma gamma globulin solution as standard. The concentration of the purified spinach calmodulin determined in this way was compared with that evaluated from its known molecular absorption coefficient; both values virtually matched. As a control, the performance of the Bradford test was also evaluated on highly purified bovine brain calmodulin. (e) The latter data seem to be a good indicator that the sample is not contaminated by complex aromatic compounds from spinach tissue.

Electron paramagnetic resonance studies

EPR spectra were recorded on a Varian X-band EPR spectrometer, model E-112, at room temperature. Rotational correlation times (nanosecond), specifying the rate of rotaional motions, of the spin label bound to spinach calmodulin were calculated from spectral data (16)

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

where w_o is the midfield line width (gauss), and h_o and h_{-1} are the respective peak heights of the mid- and high-field lines of the first derivative EPR spectrum (Fig. 2.1). This relationship is applicable for the calculation of rotational correlation times falling within the range of isotropic tumbling of nitroxide spin probes, i.e., from 10^{-10} to about 5×10^{-8} s (17). The lengths of the proxyl spin labels employed (Fig. 2.2) have been reported (18).

A proxyl spin label, characterized by a given length, was attached to the thiol group of cysteine-26 (11) on the spinach calmodulin. For labelling, calmodulin was dissolved in Tris buffer (10 mM, pH 7.0) up to a concentration of 0.2 mg calmodulin/ml buffer, then dithiothreitol, a thiol-reducing agent (19), was added to the solution, up to a final concentration of 0.2 mM. The reaction was continued under room temperature for 18 hours. The reacted sample was then labelled with the appropriate spin label by the method described (18, 20). Simply, the spin probe was added to the reduced calmodulin solution up to a final concentration of 50 μM. Extensive dialysis against Mops buffer (10 mM, pH 6.5, 0.1 M KCl) followed. Under these experimental conditions maleimide spin probes have been shown to be highly specific for free thiol groups (20).

Fig. 2.1. A standard first derivative EPR spectrum. The direction the arrow points stands for intensity increase of the magnetic field. Here w_o is the midfield line width, h_{+1}, h_o and h_{-1} stand for the respective peak heights of the lower-, mid and high-field lines. a_o is the isotropic coupling constant.

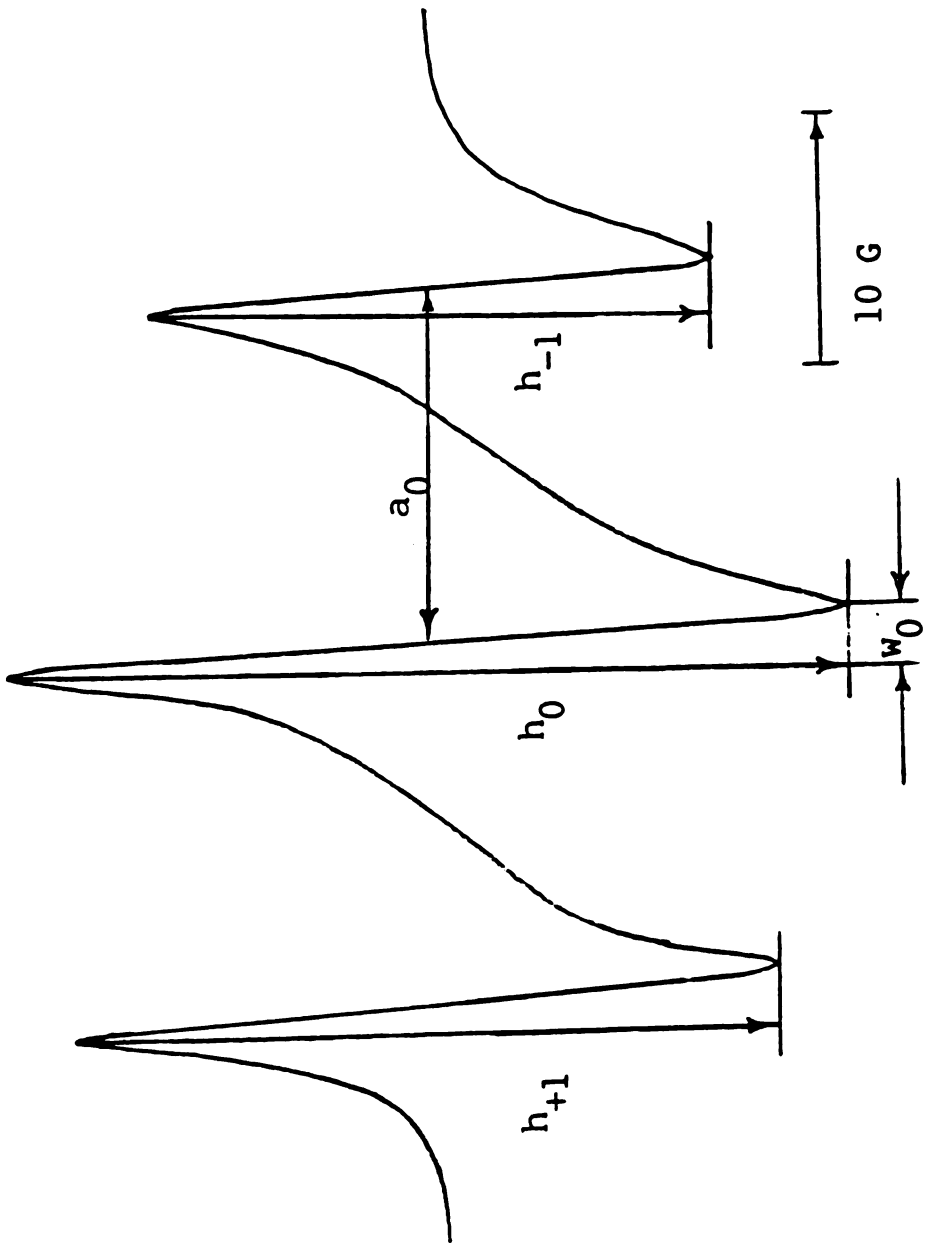
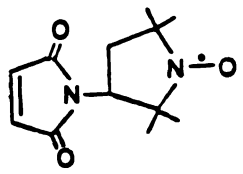
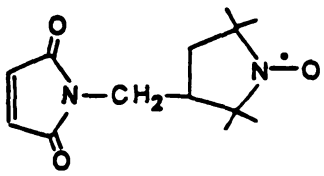
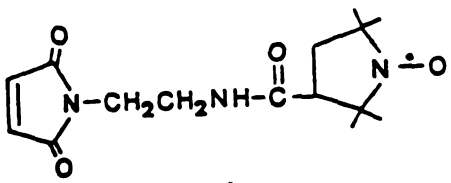
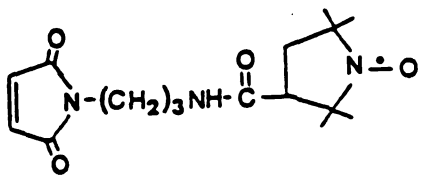
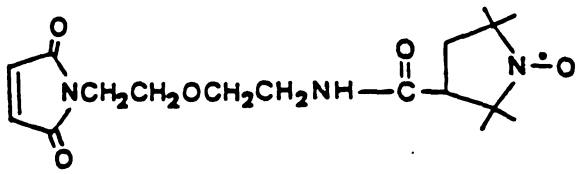

10 G
h_{-1}
a_0
h_0
w_0
h_{+1}

Fig. 2.2. Structures and lengths, D, of maleimide spin labels used in EPR experiments.

	D	D (Å)
I		4.4
II		5.7
III		9.3
IV		10.5
V		12.9

Fluorescence studies

Fluorescence experiments were performed on an SLM spectrofluorimeter, model 4800, (Urbana, IL). This instrument was interfaced with a Hewlett-Packard desk top computer, HP-85, and a plotter to aid in data acquisition and analysis. Quartz cuvettes with an optical path length of 1 cm were used. Polarization experiments were performed with Glan-Thompson calcite prism polarizers characterized by a high polarization extinction ratio. During the polarization and lifetime experiments, Corning CS 3-72 filters were used.

The steady-state fluorescence anisotropy, r, is defined as [14],

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

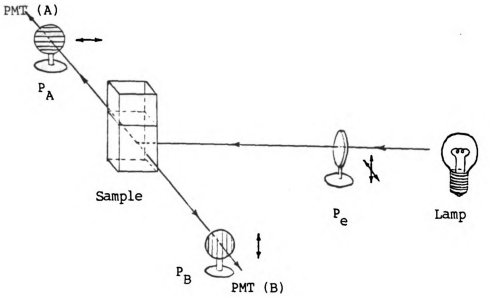
where $I_{//}$ and $I_{\perp}$ refer to the intensity of fluorescence emission measured through the emission polarizers parallel and perpendicular to the polarized plane of the exciting beam. T-format method (14) (Fig. 2.3) was used to perform anisotropy studies and Corning filters were placed in the emission beam pathways.

The anisotropy parameter, r, is related to the fluorescence lifetime, τ, and the rotational correlation time, ϕ, according to the equation (14)

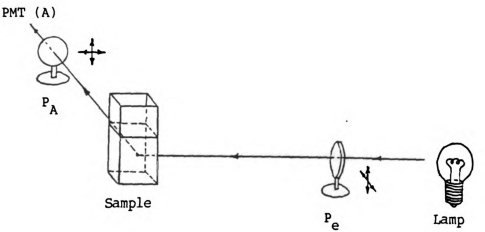
$$r = r_o(1 + \tau/\phi)^{-1} \qquad (3)$$

where r_o is the limiting anisotropy in the absence of rotational

Fig. 2.3. An illustration of T- and L-format method for measuring fluorescence anisotropy. P_e is the excitation polarizer, whose polarized plane can take two positions, one horizontal and one vertical. P_A and P_B are emission polarizers in channels A and B, respectively. PMT stands for photomultiplier. a. Each emission polarizer can take only one position. b. P_A can also take two positions.



a. T-format method



b. L-format method

diffusion. It was obtained by using Perrin plot, extrapolating the line to 0 of T/η, and the anisotropy value obtained in this way is the r_o (14). Fluorescence lifetimes were calculated from phase shift and demodulation data (14) by using a desktop computer, HP-85. 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.

At first, spinach calmodulin was reduced by dithiothreitol as described above, followed by addition of the bimane fluorescence probe (0.1 mM); this probe is highly reactive with thiol groups (21, 22). Subsequently extensive dialysis against the Mops buffer was employed after dithiothreitol reduction. Control experiments showed that both the spin and fluorescence probe-labelled spinach calmodulin had virtually the same biochemical activity as that of the unlabelled calmodulin, as judged by an enzyme assay of calmodulin-dependent phosphodiesterase activity (23).

The molar ratio of bimane probe bound per protein was found to be unity as determined from the known protein concentration and the dye's absorbance, ε_{378}=5,700 $M^{-1}cm^{-1}$ (21).

All fluorescence experiments were performed at low protein concentration to virtually exclude depolarization, energy transfer and scattering. As lifetime reference 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl) benzene was used (14). To assess the performance of the fluorescence instrumentation, control experiments with several quinine solutions were performed as lifetime standards [24]. As fluorescence anisotropy standard N-acetyl-L-tryptophanamide in propylene glycol was

used (25).

Results

EPR studies

EPR spectra of spinach calmodulin, associated with proxyl spin probes of different length, are depicted in Fig. 2.4. Qualitatively speaking, the EPR spectrum of spin probe I appears to reflect motional characteristics of a more immobilized label compared with those of spin probe V. The rotational correlation time, as determined from eq. (1), is dependent on the length of the spin label (Fig. 2.5). Apparently there exists a stepwise dependence of the value of the correlation time as the length of the spin probe increases (Fig. 2.5). Upon addition of 2 Ca^{2+} per calmodulin, the correlation times of the shortest spin probes, i.e., I and II, become faster, while those of the other three probes remain practically unchanged. At a molar ratio of 5:1 for $[Ca^{2+}]$/ [calmodulin], the rotational correlation times of all 5 spin labels virtually coincide with the correlation times determined in the absence of added Ca^{2+} (Fig. 2.5).

Upon binding of the small peptide, mastoparan, to spin-labelled calmodulin, the correlation time of the shortest spin probe, I, is significantly reduced compared with the correlation times of the longer spin probes. Apart from spin label I, the stepwise dependence of the correlation time on label length is practically unchanged (Fig. 2.5).

Fig. 2.4. First derivative EPR spectra of spin-labelled apocalmodulin (0.18 mg/ml) in Mops buffer (10 mM, pH 6.5, 0.1 M KCl), at 22° C. Following treatment with dithiothreitol, at 22°C, spinach apocalmodulin was labelled with the respective spin probe. 5 different spin probes (see Fig.1) were employed for labelling.

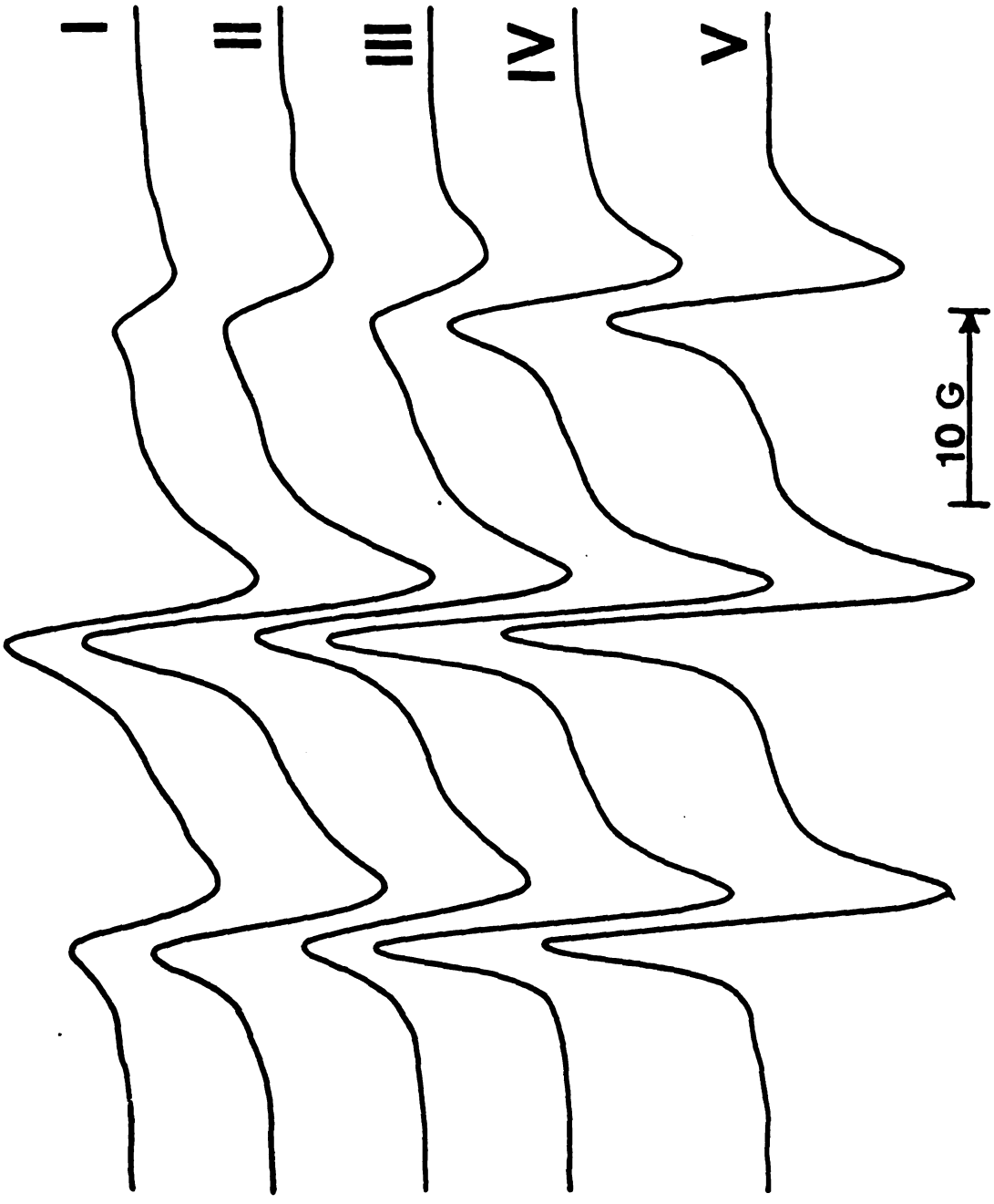
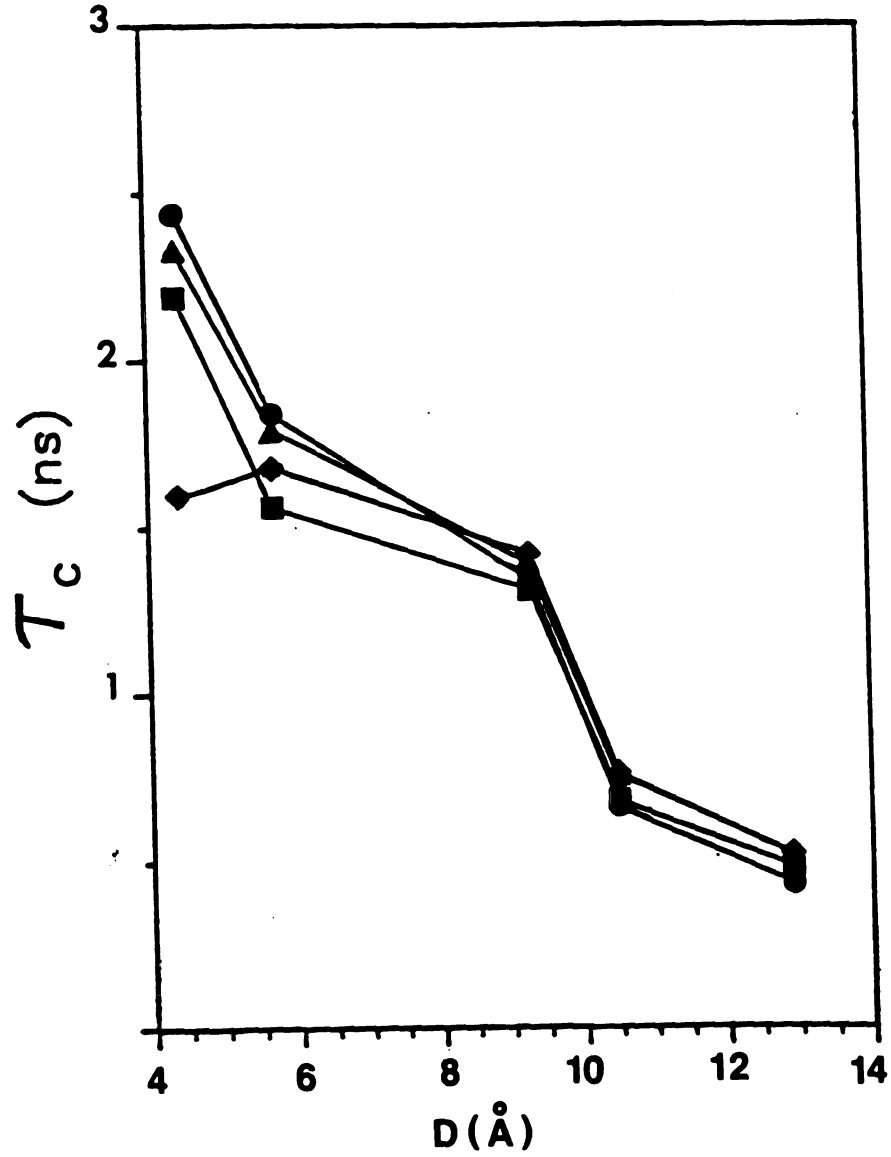

I
II
III
IV
V
10 G

Fig. 2.5. Dependence of the rotational correlation time, τ_c, of spin-labelled spinach calmodulin on spin label length, D. (●) apocalmodulin; (■) $[Ca^{2+}]$/[calmodulin] = 2:1; (▲) $[Ca^{2+}]$/[calmodulin] = 5:1; (◆) $[Ca^{2+}]$/[mastoparan]/[calmodulin] = 5:1:1. The experiments were performed in Mops buffer (10 mM, pH 6.5, 0.1 M KCl), at 22°C. The error of the correlation time is ± 0.05 ns.


3
2
1
T_c (ns)
4
6
8
10
12
14
D(Å)

Fluorescence studies

The apparent fluorescence lifetimes of the bimane-labelled calmodulin and those of the free fluorescence probe are listed in Table 2.1. The fluorescence intensity of the free dye seems to decay in a monoexponential fashion (because the τ_m and τ_p of the free probe, measured at 18 MHz and 30 MHz, were virtually identical, viz., 3.85±0.10 ns), but upon dye binding to the protein the fluorescence decay is no more monoexpotential. From a heterogeneity analysis of the raw data, the fluorescence decay of bimane-labelled calmodulin can be described as a superposition of two exponentially decaying components, a short- and a long-lived one, respectively (Table 2.1). This assumption is in accord with results from time-resolved anisotropy studies on bovine calmodulin labelled with 2,6-toluidinylnaphthalene-sulfonate; the position of this probe on the protein surface is not known (26). Upon addition of Ca^{2+} to the protein, at a molar ratio of 2:1 and 5:1, in the absence of mastoparan, the lifetime of the short-lived component remains practically unchanged, whereas the lifetime of the long-lived component appears to become shorter concomitant with an increase in [Ca^{2+}] concentration (Table 2.1).

Comparing the fluorescence parameters listed (Table 2.2), it seems that the anisotropy value decreases upon addition of 2 Ca^{2+} per protein to bimane-labelled calmodulin in solution, but at a molar ratio of 5:1 for [Ca^{2+}]/ [calmodulin], the anisotropy increases to a value slightly above that typical of calmodulin in the absence of Ca^{2+}. Upon addition of an equimolar amount of mastoparan to bimane-labelled

Table 2.1. Fluorescence lifetimes, τ_1, τ_2, and associated preexponential factors, a_1 and a_2, of bimane-labelled spinach calmodulin. These values were determined on an SLM spectrofluorimeter, model 4800, by the phase shift and demodulation methods and analyzed on a Hewlett-Packard computer, model 85. The sample was excited at 385 nm, the emission was recorded as light passing a Corning filter, CS 3-72. The calmodulin concentration was 0.18 mg/ml in Mops buffer (10 mM, pH 6.5, 0.1 M KCl), at 22°C.

sample	$\tau_{1,2}$ (ns)	preexpontial factor ($a_{1,2}$)
calmodulin	2.7	0.33
	9.5	0.67
[calmodulin]/[Ca^{2+}] = 1:2	2.9	0.34
	8.6	0.66
[calmodulin]/[Ca^{2+}] = 1:5	2.5	0.29
	7.6	0.71
[calmodulin]/[Ca^{2+}]/ [mastoparan] = 1:5:1	4.2	0.61
	10.9	0.39

Table 2.2. Molecular parameters for bimane-labelled spinach calmodulin The values, $\tau_{av.}$, were calculated from the data listed in Table 1 according to [14]. The corresponding rotational correlation times, $\phi_{av.}$, were calculated from the eq.(3) in the MATERIALS AND METHODS, using the anisotropy values, r, listed and a limiting anisotropy value of r_o = 0.2142, derived from the Perrin plot (Fig.4).

sample	$\tau_{av.}$ (ns)	$\phi_{av.}$ (ns)	r	percentage*
calmodulin	8.7	6.9	0.0949	100
[calmodulin]/[Ca^{2+}] = 1:2	7.8	5.6	0.0894	81
[calmodulin]/[Ca^{2+}] = 1:5	7.0	5.5	0.0943	80
[calmodulin]/[Ca^{2+}]/ [mastoparan] = 1:5:1	8.4	8.4	0.1072	122

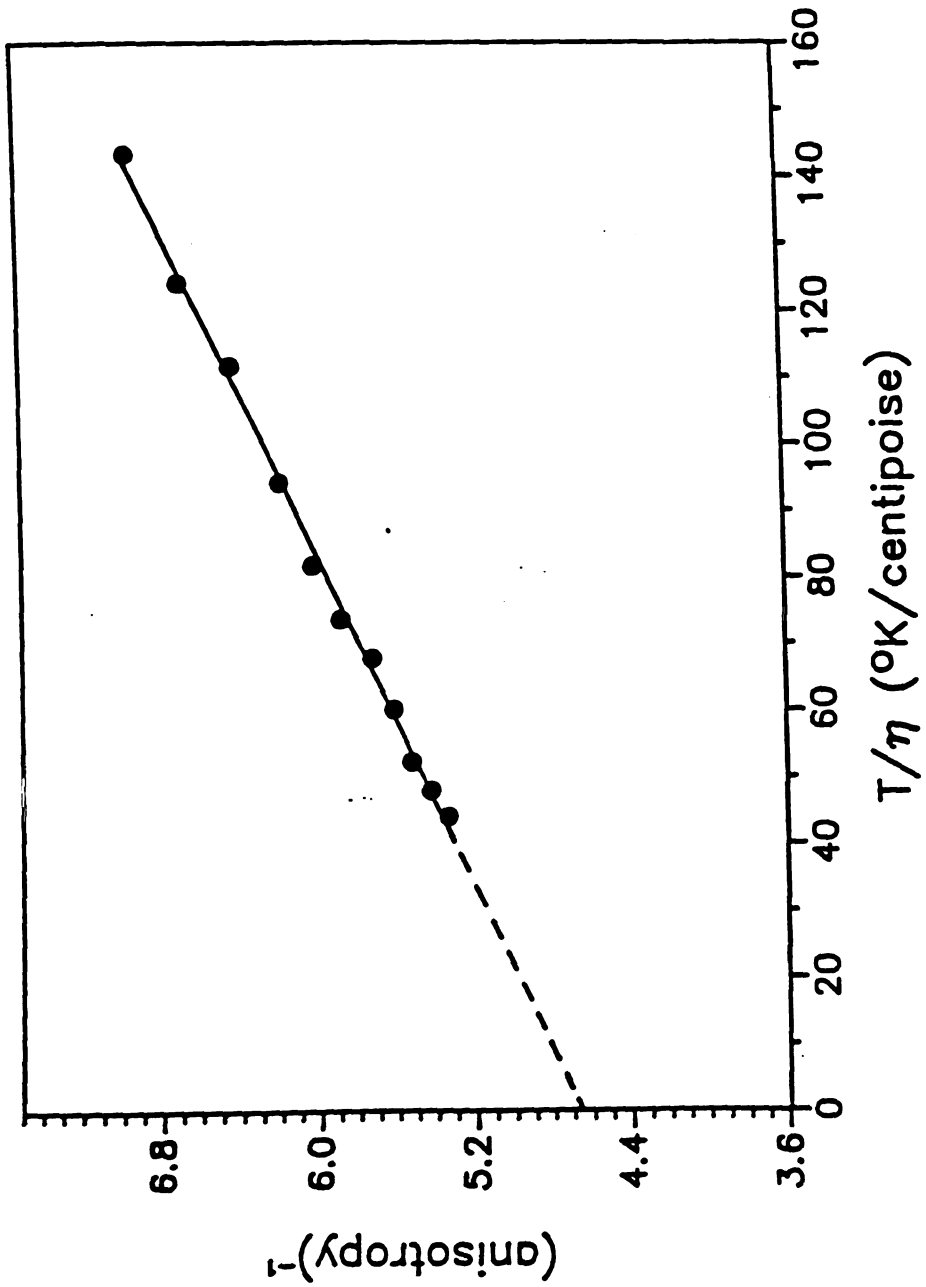
*: the rotational correlation time of calmodulin is assigned a value of 100 percent. The other percentage values are the ratios of the respective rotational correlation times to that of calmodulin.

calmodulin, the anisotropy value exceeds that ascertained for bimane-labelled calmodulin in the presence of [Ca^{2+}]/ [calmodulin] =5:1 (Table 2.2). Corresponding molecular parameters are listed for the interaction of calmodulin with the peptide (Table 2.2). By extrapolation of the anisotropy values to the ordinate in a Perrin plot (14), r^{-1} vs T/η , (Fig. 2.6), the limiting anisotropy value, r_o, can be derived.

The viscosity of the sample solution was gradually increased by adding concentrated sucrose solution. The resulting viscosity value of the aqueous sucrose mixtures was obtained from the literature [27]. The r_o value, obtained by extrapolation (Fig. 2.6), is lower than that determined for bimane-labelled S100 protein, at -20°C, in the presence of 80/20 (v/v) polypropylene glycol/water (21).

The rotational correlation times of the bimane-labelled calmodulin were calculated from eq. (3). Adding two Ca^{2+} to calmodulin in solution, the average rotational correlation time decreased to about 81 percent of that measured in the absence of Ca^{2+}. Upon application of additional 3 Ca^{2+}, the rotational correlation time remained virtually constant. On the other hand, upon mastoparan binding to calmodulin (Table 2.2), the rotational correlation time increased dramatically to about 122 percent of the value determined for apocalmodulin in solution.

Fig. 2.6. Perrin plot of spinach calmodulin whose single cysteinyl residue 26 was labelled with a fluorescence probe, monobromotrimethyl ammoniobimane. The fluorescence anisotropy value was measured by the T-format method in an SLM spectrofluorimeter, model 4800. Two CS 3-72 filters were placed in the emission path. The sample was excited at 385 nm. The viscosity, η, was increased by adding concentrated sucrose solution (1 g/ml) into the sample solution. The experiments were carried out in Mops buffer (10 mM, pH 6.5, 0.1 M KCl), at 22°C. The error in anisotropy is $\pm$ 0.0004.


(anisotropy)$^{-1}$
6.8
6.0
5.2
4.4
3.6
0
20
40
60
80
100
120
140
160
T/η (°K/centipoise)

Discussion

Calmodulin, like other globular proteins, is a dynamic system undergoing internal motions on a time scale as fast as picoseconds (7). These internal motions and their proper time-correlation are probably crucial for protein functioning (28). In such a dynamic system there exist a multitude of conformational substates which are accessible by rapid structural fluctuations of the protein. Ligand-triggered structural perturbations, e.g., binding of Ca^{2+} to high-affinity sites of calmodulin, may therefore be transmitted in a flexible macromolecule (29) over a certain distance. Indeed, we have demonstrated in this article that a response to ligand-induced perturbations is elicited at a specific site of calmodulin.

The dynamic interplay of calmodulin's constituent parts can be better understood in terms of calmodulin's crystal structure. X-ray diffraction experiments on bovine calmodulin indicate that the protein has the shape of a dumbbell (3) with an overall length of about 6.2 nm in the presence of calcium (4). The calcium-binding regions I and II are located in the lobe containing the amino terminus, whereas the high-affinity regions III and IV (1) reside in the lobe with the carboxyl terminal end. The two lobes are interconnected by an eight-turn-helix.

Spinach calmodulin harbors a single cysteinyl residue at site 26 which is located in region I of the protein, replacing a threonyl residue present in bovine brain calmodulin (11). The rotational correlation times of spin-labelled spinach calmodulin are those of

rapidly tumbling nitroxide labels. This suggests that the binding site, i.e., cysteine-26, is not buried inside the protein but seems to be exposed at the protein's surface. The label tested seems to reside in a polar microenvironment as evidenced by an isotropic coupling constant, a_o, of 16.0 $\pm$0.2 gauss; this value was unaffected by the presence or absence of Ca^{2+}. The isotropic coupling constant measured is comparable to that found for nitroxide in ethanol as solvent [30]. A constant label microenvironment is also indicated by our fluorescence studies, where the fluorescence maximum (wavelength and intensity) did not change in response to the application of Ca^{2+} and a peptide target.

The rotational correlation times measured for spin-labelled spinach apocalmodulin show that the shorter spin probes, i.e., I and II, have less motional freedom than the longer spin labels. However, binding of 2 Ca^{2+} per protein somewhat alleviates the motional restrictions imposed by the microenvironment around the shorter spin probes. Monitored at cysteine-26 in region I, located in the amino terminal lobe of spinach calmodulin, these motional changes probably do not result from direct interactions of the cations with this site because the first two Ca^{2+} added to calmodulin seem to bind to the high-affinity binding regions (1) located in the carboxyl terminal lobe of the molecular dumbbell. Rather, as a result of calcium coordination at the high-affinity sites, structural perturbations are produced which are being transmitted over a considerable distance to the other lobe. Upon further addition of Ca^{2+} to the protein, up to a molar ratio of 5:1, calcium binding takes place in regions I and II, i.e., in the

immediate vicinity of the spin probe. Consistent with our results on the motional characteristics of the shorter spin labels one wouold expect a decrease in the label's mobility when calcium is specifically anchored in its coordination cage of region I when compared with those without calcium binding at any sites at all. Our findings about the calcium dependence of spin label motion at site 26 are also in accord with spectroscopic experiments indicating that Ca^{2+} binding by bovine calmodulin takes place in at least two distinct stages (31).

As monitored at site 26, marked motional changes are reported by the shortest spin label upon binding of mastoparan to calmodulin. The amphiphilic tetradecapeptide, mastoparan, has an association constant of 3.3 nM^{-1} with calmodulin bound with calcium (32), and undergoes a conversion from a conformation which is low in -helix to a highly helical structure (33) upon formation of the calmodulin-mastoparan complex, probably in region III of calmodulin's C-terminal half [31], when Ca^{2+} is present. This binding region seems to partially match that of cyclic nucleotide phosphodiesterase because the wasp venom, mastoparan, is a potent inhibitor of this enzyme (34). Compared with the rotational correlation time of the shortest spin label in apocalmodulin or with that in the presence of Ca^{2+}, this time is reduced by about 30 percent when spinach calmodulin interacts with a partner peptide, mastoparan. Apparently a specific response is elicited at the cysteine-26 site following transmission of structural perturbations triggered in the specific target region where calmodulin directly communicates with its partner protein. Our observations are in accord with NMR data on the binding of a 27-residue peptide (denoted

M13) to bovine calmodulin. Derived from skeletal muscle myosin light chain kinase, M13 peptide binding affected many NMR resonances arising from residues in both moieties of calmodulin (35).

The fluorescence data (anisotropy, apparent lifetimes) obtained from experiments on the bimane probe, attached to cysteine-26 of spinach calmodulin, are complementary to results derived from EPR measurements. The former data can be interpreted as follows: the average rotational correlation time seems to be associated with the protein's rotational motion, probably that arising from a major portion of the calmodulin molecule. When two Ca^{2+} are added to calmodulin in solution, the protein's rotational motion is enhanced compared with that in the absence of Ca^{2+}, consistent with the compaction reported (36); the rotational motion remains unchanged upon further addition of Ca^{2+}, which bind to regions I and II of the protein. Following peptide binding to calmodulin, the rotational motion of the protein-ligand complex slows down because the total volume of the complex is larger than that of the apoprotein. Calmodulin (M = 16 800 for bovine calmodulin) has a rotational correlation time of about 6.8 ns, assuming a partial specific volume of 0.73 cm^3/g and a hydration of 0.2, respectively. Given this simple picture of a spherical molecule, it appears that the relatively short bimane fluorescence probe at cysteine-26 serves as a reporter group for the rotational motion of the protein, characterized by an average rotational correlation time, either in the absence or presence of Ca^{2+}. When a specific complex is formed between calmodulin and mastoparan, the correlation time for the overall rotation of the complex is becoming slower, as monitored by the

bimane reporter group at cysteine-26.

In conclusion, we have demonstrated that a ligand-triggered, Ca^{2+}-dependent response can be monitored at a site distant from the trigger region. Moreover, the methodology introduced of exploiting the presence of a single cysteinyl residue in spinach calmodulin may be beneficially applied for the analysis of more detailed dynamic aspects of calmodulin in relation to specific partner proteins.

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LIST OF REFERENCES

1. Klee, C.B. and Vanaman, T.C., (1982) Calmodulin. Adv. Protein Chem. 35: 213-321.

2. Kretsinger, R., (1975) Hypothesis: Calcium modulated proteins contain EF hands. in: Calcium Transport in Contraction and Secretion (Carafoli, E., Clementi, F., Drabikowski, W. & Margreth, A., eds.), North-Holland Publ., Amsterdam. pp.469-478.

3. Babu, Y.S., Sack, J.S., Greenhough, T.J., Bugg, C.E., Means, A.R., and Cook, W.J., (1985) Three-dimensional structure of calmodulin. Nature (London) 315: 37-40.

4. Seaton, B.A., Head, J.F., Engelman, D.M., and Richards, F.M., (1985) Calcium-induced increase in the radius of gyration and maximum dimension of calmodulin measured by small-angle X-ray scattering. Biochemistry 24: 6740-6743.

5. Sellinger-Barnette, M. and Weiss, B., (1984) Interaction of various peptides with calmodulin. Adv. Cycl. Nucleot. Protein Phosphoryl. Res. 16: 261-276.

6. Weis, C. and Haug, A., (1987) Aluminum-induced conformational changes in calmodulin alter the dynamics of interaction with melittin. Arch. Biochem. Biophys. 254: 304-312.

7. Karplus, M. and McCammon, J. A., (1981) The internal dynamics of globular proteins. CRC Crit. Rev. Biochem. 9: 293-349.

8. Chou, K.C. and Kiang, Y.S., (1985) The biological functions of low-frequency vibrations (phonons) 5. A phenomenological theory. Biophys. Chem. 22: 219-235.

9. Elber, R. and Karplus, M., (1987) Multiple conformational states of proteins: a molecular dynamics analysis of myoglobin. Science 235: 318-321.

10. Beece, D., Eisenstein, L., Frauenfelder, H., Good, D., Marden, M.C., Reinisch, L., Reynolds, A.H., Sorensen, L.B., and Yue, K.T., (1980) Solvent viscosity and protein dynamics. Biochemistry 19. 5147-5157.

11. Vanaman, T. C., (1983) Chemical approaches to the calmodulin system. Methods Enzymol. 102: 296-310.

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

13. Watterson, D.M., Iverson, D.B. and Van Eldik, L.J., (1980) Spinach

calmodulin: isolation, characterization and comparison with vertebrate calmodulins. Biochemistry 19: 5762-5768.

14. Lakowicz, J.R., (1984) Principles of Fluorescence Spectroscopy. Plenum Press, New York.

15. Bradford, M.M., (1976) A rapid and sensitive method for the quatitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

16. Keith, A., Bulfield, G. and Snipes, W., (1970) Spin-labeled Neurospora mitochondria. Biophys. J. 10: 618-629.

17. Mehlhorn, R.J. and Keith, A.D., (1972) in: Membrane Molecular Biology (Fox, C.F. & Keith, A.D., eds.), Sinauer Assoc., Stamford, CT. pp. 192-227.

18. Lai, C.-S., Tooney, N.M. and Ankel, E.G., (1984) Spin label studies of sulfhydryl environment in plasma fibronectin. FEBS lett. 173: 283-286.

19. Cleland, W.W., (1964) Dethiothreitol, a new protective reagent for SH groups. Biochemistry 3: 480-482.

20. Lai, C.-S., and Tooney, N.M., (1984) Electron spin resonance spin label studies of plasma fibronectin: effect of temperature. Arch. Biochem. Biophys. 228: 465-473.

21. Kosower, N.S., Kosower, E.M., Newton, G.L., and Ranney, H.M., (1979) Bimane fluorescent labels: labeling of normal human red cells under physiological conditions. Proc. Natl. Acad. Sci. (U.S.) 76: 3382-3386.

22. Baudier, J., Glasser, N. and Duportail, G., (1986) Bimane- and acrylodan- labeled S100 proteins: role of cysteines-85 and -84 in the conformation and calcium binding properties of S100 and S100b( ) proteins. Biochemistry 25: 6934-6941.

23. Teo, T.S., Wang, T.H. and Wang, J.H., (1973)Purification and properties of the protein activator of bovine heart cyclic adenosine 3', 5'-monophosphate phosphodiesterase. J. Biol. Chem. 248: 588-595.

24. Chen, R.F., (1974) Fluorescence lifetime reference standards for the range 0.189 to 115 nanoseconds. Anal. Biochem. 57: 593-604.

25. Lakowicz, J.R. and Weber, G., (1980) Nanosecond segmental mobility of tryptophan residues in proteins observed by lifetime-resolved fluorescence anisotropies. Biophys. J. 32: 591-601.

26. Steiner, R.F. and Norris, L., (1987) Rotational modes of

Ca^{2+}-liganded calmodulin, as determined by time-domain fluorescence. Biophys. Chem. 27: 27-38.

27. Weast, R.C. (1983/1984) CRC Handbook of Chemistry and Physics, p. D-266, CRC Press, Boca Raton, FL.

28. Careri, G. (1984) Order and Disorder in Matter, Benjamin/Cummings Publ., Menlo Park, CA.

29. Lambooy, P.K., Steiner, R.F., and Sternberg, H., (1982) Molecular dynamics of calmodulin as monitored by fluorescence anisotropy. Arch. Biochem. Biophys. 217: 517-528.

30. Morrisett, J.D., (1976) The use of spin labels for studying the structure and function of enzymes. in: Spin Labeling: Theory and Applications, (Berliner, L.J., ed.), Academic Press, New York, NY. pp. 273-338.

31. Muchmore, D.C., Malencik, D.A. and Anderson, S.R., (1986) ^{+}H NMR studies of mastoparan binding by calmodulin. Biochem. Biophys. Res. Commun. 137: 1069-1078.

32. Malencik, D.A. and Anderson, S.R., (1983) Binding of hormones and neuropeptides to calmodulin. Biochemistry 22: 1995-2001.

33. Cachia, P.J., Van Eyk, J., Ingraham, R.H., McCubbin, W.D., Kay, C.M., and Hodges, R.S., (1986) Calmodulin and troponin C: a comparative study of the interaction of mastoparan and troponin I inhibitory peptide [104-115]. Biochemistry 25: 3553-3562.

34. Barnette, M.S., Daly, R., and Weiss, B., (1983) Inhibition of calmodulin activity by insect venom peptides. Biochem. Pharm. 32: 2929-2933.

35. Klevit, R.E., Blumenthal, D.K., Wemmer, D.E. and Krebs, E.G., (1985) Interaction of calmodulin and a calmodulin-binding peptide from myosin light chain kinase: major spectral changes in both occur as the result of complex formation. Biochemistry 24: 8152-8157.

36. LaPorte, D.C., Keller, C.H., Olwin, B.B. and Storm, D.R., (1981) Preparation of a fluorescent-labeled derivative of calmodulin which retains its affinity for calmodulin binding proteins. Biochemistry 20: 3965-3972.

CHAPTER III

DYNAMIC PROPERTIES OF CALMODULIN IN RESPONSE TO ALUMINUM

Shixing Yuan and Alfred Haug

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As monitored by a fluorescence dye attached to the single cysteinyl residue 26 of spinach calmodulin, application of aluminum ions to calmodulin, in the presence of saturating Ca^{2+}, results in motional changes of the fluorescence probe different from those observed in the absence of aluminum. In the presence of aluminum, the extent of hydrophobicity of the probe's microenvironment resembles that observed in apocalmodulin. The average rotational correlation times of calmodulin are dependent on temperature and the type of cation, i.e., calcium and aluminum. Energy transfer studies from the single tryptophanyl residue of a calmodulin target peptide, mastoparan-X, to the fluorescence probe as acceptor residing at cysteine-26 of spinach calmodulin, show a higher transfer efficiency in the aluminum-altered protein. We therefore conclude that structural and dynamic changes take place in aluminum-altered spinach calmodulin, even in the presence of saturating Ca^{2+} concentrations.

Introduction

Aluminum, the most abundant metal in the earth's crust, had 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 available for plants (2, 3). Besides natural soil acidity, anthropogenic perturbation of hydrogeo-chemical 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_r=16,000-17,000). This acidic protein has been highly conserved during evolution of eukaryotic cells where it participates 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 for 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.

Materials and Methods

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 a protein assay kit were purchased from Bio-Rad Laboratories (Richmond, CA).

Calmodulin was isolated from fresh spinach using methods previously described (14, 17, 18). Spinach calmodulin obtained in this manner was found to be free of tryptophan as judged by UV absorbance and lack of fluorescence emission at 340 nm following excitation with 295 nm light. In addition, the protein's UV absorption spectrum showed a partially resolved vibrational structure typical of that known for bovine brain calmodulin (10). Furthermore, analysis of the spinach calmodulin preparation on 15 percent polyacrylamide gels followed by Coomassie blue staining indicated a single band migrating at an apparent molecular weight of 17,000, this result being consistent when up to 45

ug protein were loaded per gel lane.

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 reduction 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 for about 2 hours with the sample as described (19). Finally, the reaction mixture was extensively dialyzed against Mops buffer (10 mM at pH 6.5 and 0.1 M KCl), which was used in all experiments. The molar ratio of AEDANS bound per protein was found to be 0.95 to 1, as determined by dye absorbance ($\mathcal{E}$=6,100 cm^2 mM^{-1} at 337 nm) (20) and Bradford assay (21), respectively. Unless indicated otherwise, the protein concentrations employed were 10 uM. To occupy all four Ca^{2+} binding domains of calmodulin, Ca^{2+} ions were added to the protein at a molar ratio of 5:1, to achieve saturating conditions.

As described previously (15,18), fluorescence experiments (anisotropy, lifetime) were performed on an SLM spectrofluorimeter, model 4800, manufactured by SLM Instruments, Inc. (Urbana, IL). For each assay a Beckman cuvette with 1 cm optical pathlength and a sample volume of 2.5 ml was used. Furthermore, at the low protein concentrations used, the anisotropy and the lifetime were independent of protein concentration (22). Each data point represents the average value for 100 counts per sample which, in turn, was four times replicated.

Fluorescence energy transfer was evaluated between a donor, D, viz., the single tryptophanyl residue on mastoparan-X (M = 1,557) and an acceptor, A, viz., the fluorescence label, AEDANS, residing at the single cysteinyl residue, Cys-26 (23), 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 (24), 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 and that in the absence of the acceptor. The ratio Q_{DA}/Q_D can be evaluated from corresponding fluorescence intensities, 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 (22), are measured upon excitation of the donor at 299 nm to minimize the excitation of tyrosin. The quantities, f_D and f_{DA}, are the fluorescence intensities of the donor, measured in the absence of the acceptor, and in the presence of the acceptor within a wavelength range free from acceptor emission, respectively. Subsequently the intensity ratios were averaged, thus yielding $[f_{DA}/f_D]_{av}$. $[D]_D$ 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, D, to the acceptor, A, 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

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

Here, 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 (25). Since the aromatic ring systems of the donor (tryptophan) (22) and the acceptor (AEDANS) have two linear transition moments each, the assumption of random orientation appears to be reasonable. Moreover, n is the refractive index and J_{DA} is the spectral overlap integral defined as

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

where ε_A is the extinction coefficient of the acceptor in $M^{-1}cm^{-1}$; λ is the wavelength in nm.

Four kinds of samples have to be prepared for these energy transfer studies: (a) Ca-calmodulin plus mastoparan (neither donor nor acceptor present), (b) Ca-calmodulin plus mastoparan-X (donor present, D); (c) AEDANS-labelled Ca-calmodulin plus mastoparan (acceptor present, A);

and (d) AEDANS-labelled Ca-calmodulin plus mastoparan-X (both donor and acceptor present, DA).

Results

Our experiments show that the emission of the AEDANS-spinach calmodulin conjugate reaches a maximum at 490 nm and is considerably blue-shifted from the emission maximum of 520 nm reported for the aqueous free probe (20). An emission maximum observed at 490 nm corresponds to that reported for AEDANS dissolved in 60% ethanol/water (20). When covalently bound to spinach calmodulin, the probe apparently resides in an environment less accessible to water. Increased rigidity or further water removal from 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 3.1 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 upon calcium binding (26). At a molar ratio of [Al]/[calcium saturated calmodulin] = 3:1, the rotational

correlation time significantly increases relative to the time determined in the absence of aluminum. The rotational correlation times were calculated from eq.(2) using an r_o value of 0.0910 ± 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 (20). Since our instrument does not permit measurements of time-resolved anisotropy decays, an average rotational correlation time had to be calculated (Table 3.1).

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. This kind of peptide is known to bind calmodulin with high affinity in a calcium-dependent manner (16). The wavelength region of the fluorescence emission band of tryptophan shows excellent overlap with that of the acceptor's absorption spectrum, AEDANS-conjugated cysteine (Fig. 3.1), 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 eq.(7). From the reported values of the donor quantum yield, Q_D, of 0.155 (27), a refractive index of n = 1.334, corresponding to that of the 0.1 M KCl solution at room temperature (28), and an orientational factor of $k^2 = 2/3$, the Förster critical

Table 3.1. Fluorescence properties of AEDANS-labelled spinach calmodulin at various cation concentrations

sample	r	a_1^* / a_2	τ_1^* / τ_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

* the preexponential factors, a_1 and a_2, and the corresponding lifetimes, τ_1 and τ_2, were analyzed on the basis of a biphasic decay. The error in lifetimes is $\pm$ 0.07 ns.

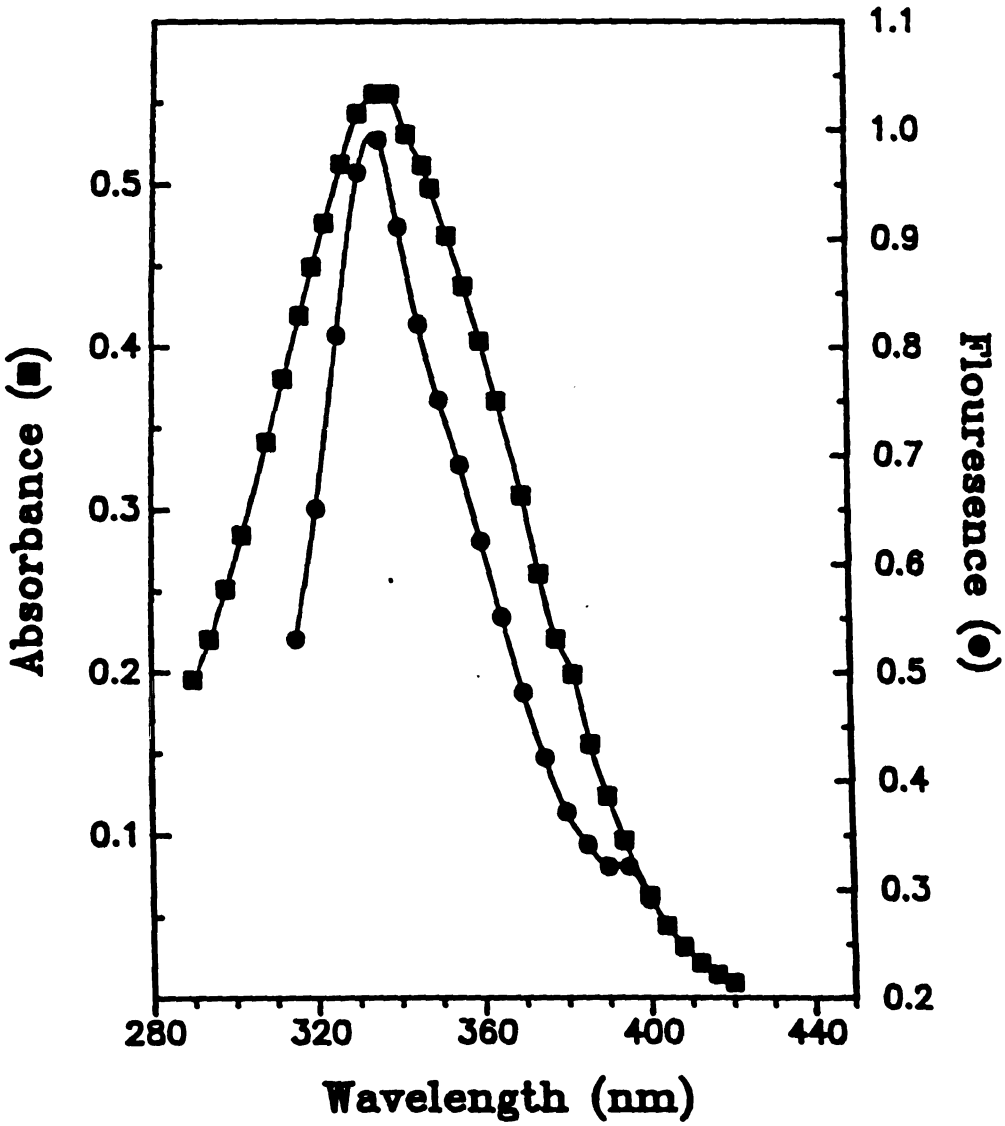
r is the fluorescence anisotropy (error $\pm$ 0.0004) measured by T-format method as indicated in legend to Figure 2, except at room temperature.

τ_{av} are average lifetimes calculated from the relationship (22)

$$\tau_{av} = (a_1\tau_1^2 + a_2\tau_2^2)/(a_1\tau_1 + a_2\tau_2)$$

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

Figure 3.1 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-morpholino-propane 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.


Absorbance (■)
Flouresence (●)
Wavelength (nm)
0.1
0.2
0.3
0.4
0.5
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
280
320
360
400
440

distance, R_o, can be determined (Table 3.2). The Förster critical distance, R_o, is in accord with that reported for the radiationless energy transfer from Trp-19 of melittin to AEDANS-labelled troponin C at Cys-98, viz., R_o = 23.6 A^o (29). 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 3.2). The efficiency of energy transfer between donor and acceptor was higher when aluminum was present in the sample, resulting in a distance of 29.4 A^o. In the absence of aluminum, this distance was 30.9 A^o (Table 3.2). Mastoparan-X probably binds to calmodulin's region III, when calcium is present (30). 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.

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 (Fig. 3.2). The anisotropy decreased as the temperature increased; however, in the presence of aluminum ions, the anisotropy value was higher at all temperatures tested. Moreover, temperature-induced structural changes of calmodulin are also reflected in molecular parameters representative for those of the protein (Table 3.3). The molecular parameters were evaluated at two temperatures. At 18.2°C, the rotational correlation time, 29.1 ns, measured for AEDANS-labelled spinach calmodulin in the presence of calcium, is in general accord with values measured at different wavelengths for bovine calmodulin (31). At 18.2°C, the

Table 3.2. 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_{AB})	R_o (A^o)	R (A^o)
calmodulin + 5 Ca^{2+}	0.194 ± 0.014	6.50×10^{13}	23.9	30.3
calmodulin +5Ca^{2+}+ 3Al	0.219 ± 0.009	6.50×10^{13}	23.9	29.4

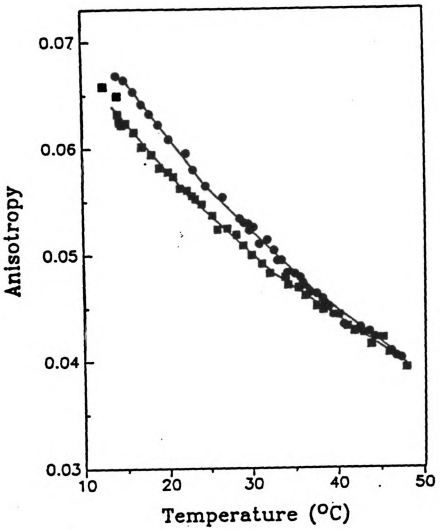
R_o is the Förster critical distance, as calculated from eq. 6. R is the distance between donor and acceptor molecule, as calculated from eq. 5. The experiments were performed at room temperature.

Table 3.3. Molecular parameters of AEDANS-labelled spinach calmodulin

sample	temperature (C^o)	anisotropy	τ_{av} (ns)	ϕ_{av} (ns)
calmodulin $+5Ca^{2+}$	18.2	0.0571 ± 0.0003	17.3	29.1
	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 3.1

Figure 3.2. 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 μM. (■), [calmodulin]:[Ca^{2+}] =1:5; (●), [calmodulin]: [Ca^{2+}]:[aluminum] = 1:5:3.


0.07
0.06
0.05
0.04
0.03
Anisotropy
10
20
30
40
50
Temperature (°C)

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 3.3).

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-helix (32). 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 (33, 34). These conformational substates are in part dependent on the viscosity of the solvent (35). Our understanding of the molecular dynamics of proteins is in its infancy, however, evidence is accumulating that at least some of these conformational substates are crucial for protein function (34, 36).

Indeed, we have demonstrated in this article that aluminum ions perturb the conformation of calmodulin existing in the presence of calcium ions. This is illustrated by our findings that application of aluminum ions to [calcium]/[calmodulin] = (5:1) alters the fluorescence probe's motional properties different from those with aluminum absent. Moreover, the probe seems to reside in a hydrophobic area which is not influenced by the binding of calcium or aluminum. Our data on the

influence of temperature on the fluorescence anisotropy suggest that application of aluminum to spinach calmodulin produces a calmodulin structure 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, changes may reflect rearrangements in the solid- and liquid-like microdomain structure of the protein (36). 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).

Acknowledgement

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LIST OF REFERENCES

1. Hartwell, B.L., and Pember, F.R., (1918) The presence of aluminum as a reason for the difference in the effect of so-called acid soil on barley and rye. Soil Sci. 6: 259-279.

2. Foy, C.D., Chaney, R.L., and White, M.C. (1978) The physiology and metal toxicity in plants. Ann. Rev. Plant Physiol. 29: 511-566.

3. Haug, A, (1984) Molecular aspects of aluminum toxicity. CRC Crit. Rev. Plant Sci. 1: 345-373.

4. Stumm, M., Morgan, J.J., and Schnoor, J.L., (1983) Saurer Regen, eine Folge der Storung hydrogeochemischer Kreislaufe. Naturwiss. 70: 216-223.

5. Evans, L.S., (1982) Biological effects of acidity in precipitation on vegetation: A review. Environ. Exper. Bot. 22: 155-169.

6. Ganrot, P.O., (1986) Metabolism and possible health effects of aluminum. Environ. Health Perspect. 65: 363-441.

7. Vierstra, R., and Haug, A., (1978) The effects of Al^{3+} on the physical properties of membrane lipids in Thermoplasma acidophilum. Biochem. Biophys. Res. Comm. 84: 138-144.

8. Suhayda, C.G., and Haug, A., (1986) Organic acids reduce aluminum toxicity in maize root membranes. Physiol. Plant. 68: 189-195.

9. Deleers, M., Servais, J.P., and Wulfert, E., (1986) Neurotoxic cations induce membrane rigidification and membrane fusion at micromolar concentrations. Biochim. Biophys. Acta 855: 271-276.

10. Klee, C.B., and Vanaman, T.G., (1982) Calmodulin. Adv. Protein Chem. 35: 213-321.

11. Siegel, N., and Haug, A., (1983) Aluminum interaction with calmodulin: evidence for altered structure and function from optical and enzymatic studies. Biochim. Biophys. Acta 744: 35-45.

12. Siegel, N., and Haug, A., (1983) Calmodulin-dependent formation of membrane potential in barley root plasma membrane vesicles: A biochemical model of aluminum toxicity in plants. Physiol. Plant. 59: 285-291.

13. Siegel, N., and Haug, A., (1983) Aluminum-induced inhibition of calmodulin-regulated phosphodiesterase activity: enzymatic and optical studies. Inorgan. Chim. Acta 79: 230-231.

14. Suhayda, C.G., and Haug, A., (1985) Citrate chelation as a potential mechanism against aluminum toxicity in cells: the role of

calmodulin. Canad. J. Biochem. Cell Biol. 63: 1167-1175.

15. Weis, C., and Haug, A., (1987) Aluminum-induced conformational changes in calmodulin alter the dynamics of interaction with melittin. Arch. Biochem. Biophys. 254: 304-312.

16. Cox, J.A., Comte, M., Fitton, J.E., and DeGrado, W.F., (1985) The interaction of calmodulin with amphiphilic peptides. J. Biol. Chem. 260: 2527-2534.

17. Caldwell, C.R., and Haug, A., (1981) Affinity chromatographic isolation of calmodulin from bovine brain acetone powder. Anal. Biochemistry 116: 325-330.

18. Yuan, S., and Haug, A., (1988) Ligand-triggered conformational perturbations elicit changes at the single cysteinyl residue of spinach calmodulin. Eur. J. Biochem. 175: 119-124.

19. Wu, C.W., and Stryer, L., (1972) Proximity relationships in rhodopsin. Proc. Natl. Acad. Sci. (U.S.) 69: 1104-1108.

20. Hudson, E.N., and Weber, G., (1973) Synthesis and characterization of two fluorescent sulfhydryl reagents. Biochemistry 12: 4154-4161.

21. Bradford, M.M., (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

22. Lakowicz, J.R., (1984) Principles of Fluorescence Spectroscopy. Plenum Press, New York.

23. Vanaman, T.C., (1983) Chemical approaches to the calmodulin system. Methods Enzymol. 102: 296-310.

24. Fairclough, R.F., and Cantor, C.R., (1977) The use of singlet-singlet energy transfer to study macromolecular assemblies. Methods Enzymol. 48: 347-379.

25. Eisinger, J., (1969) Intramolecular energy transfer in adrenocorticotropin. Biochemistry 8: 3902-3915.

26. Seamon, K.B., (1980) Calcium- and magnesium-dependent conformational states of calmodulin as determined by NMR. Biochemistry 19: 207-215).

27. Steiner, R.F., Marshall, L., and Needleman, D., (1986) The interaction of melittin with calmodulin and its tryptic fragments. Arch. Biochem. Biophys. 246: 286-300.

28. Weast, R.C., (1983) Handbook of Chemistry and Physics, CRC Press,

Boca Raton, FL, p. D-24.

29. Steiner, R.F., and Norris, L., (1987) The interaction of melittin with troponin C. Arch. Biochem. Biophys. 254: 342-352.

30. Muchmore, D.C., Malencik, D.A., and Anderson, S.R., (1986) $^{+}$H NMR studies of mastoparan binding by calmodulin. Biochem. Biophys. Res. Commun. 137: 1069-1078.

31. LaPorte, D.C., Keller, C.H., Olwin, B.B., and Storm, D.R., (1981) Preparation of a fluorescent-labeled derivative of calmodulin which retains its affinity for calmodulin binding proteins. Biochemistry 20: 3965-3972.

32. Babu, Y.S., Sack, J.S., Greenhough, T.J., Bugg, C.E., Means, A.R., and Cook, W.J., (1985) Three-dimensional structure of calmodulin. Nature (London) 315: 37-40.

33. Careri, G., (1984) Order and Disorder in Matter. The Benjamin/ Cummings Publ., Menlo Park, CA.

34. Karplus, M., and McCammon, J.A., (1981) The internal dynamics of globular proteins. CRC Crit. Rev. Biochem. 9: 293-349.

35. Beece, D., Eisenstein, L., Frauenfelder, H., Good, D., Marden, M.C., Reinisch, L., Reynolds, A.H., Sorensen, L.B., and Yue, K.T., (1980) Solvent viscosity and protein dynamics. Biochemistry 19: 5147-5157.

36. Elber, R., and Karplus, M., (1987) Multiple conformational states of proteins: A molecular dynamics analysis of myglobin. Science 235: 318-321.

CHAPTER IV

FRICTIONAL RESISTANCE TO MOTIONS OF BIMANE-LABELLED SPINACH CALMODULIN IN RESPONSE TO LIGAND BINDING

Shixing Yuan and Alfred Haug

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The single cysteinyl residue 26 of spinach calmodulin was labelled with the thiol-specific bimane fluorescence probe. Following application of stoichiometric quantities of Ca^{2+} or aluminum ions to the protein, temperature-dependent fluorescence changes (anisotropy, lifetime) could be monitored via the label. From these data the Y-function could be constructed which, as a function of temperature, seems to consist of two linear regions which intersect at the critical temperature, T_c. From the Y-function the thermal coefficient, b(T), of the frictional resistance to fluorophore rotation could be determined. b(T) was dependent on the type and stoichiometry of ligand(s) bound to calmodulin. Changes of the thermal coefficient apparently resulted in part from ligand-triggered structural perturbations transmitted over a considerable distance to calmodulin region I, the site of the fluorophore.

Introduction

Calmodulin is a small (M_r=16,000-17,000), acidic protein, which mediates Ca^{2+}-dependent regulatory processes in cells. An intriguing feature of calmodulin is its capability to interact with and stimulate numerous specific enzymes (1). The biochemically active form appears to be $(Ca^{2+})_4$-calmodulin which has a pronounced tendency to bind to a basic amphiphilic-helix in a variety of target peptides (2). The protein's intrinsic flexibility probably contributes to its versatility (3). Thanks to its flexibility and associated rapid local motions, calmodulin can apparently control and thus correlate molecular events (4, 5), e.g., those triggered by specific ligands bound to specific locations. These motions and associated conformational changes are thought to be crucial for its biochemical activity (5).

To analyze the conformational changes, a small fluorophore attached to the protein may be used. For example, by measuring the temperature dependence of fluorescence polarization and lifetime of the probe, the Y-function can be established (6). This function describes the frictional resistance associated with conformational changes to the probe's rotational motions.

Conformational changes at a defined locus on calmodulin may be the result of transfer of structural perturbations initially generated by ligands bound to protein regions distant from or close to the defined locus (7). Examination of these kinds of ligand-triggered conformational changes via a fluorophore requires studies of the relative motions of calmodulin's constituent regions.

To monitor ligand-triggered conformational changes through a fluorophore, we therefore selected spinach calmodulin which harbors a single cysteinyl residue at site 26, i.e., region I of the polypeptide chain (7, 8). Our results indicate that through a strategically anchored fluorophore, conformational changes could be determined in response to ligand-triggered structural perturbations.

Materials and Methods

The fluorescence probe monobromotrimethylammoniobimane was purchased from Calbiochem Company (San Diego, CA) under the commercial name of thiolyte MQ. Phenyl-Sephrose CL-4B was obtained from Pharmacia Company (Uppsala, Sweden).

Purification of spinach calmodulin

Spinach calmodulin was purified from fresh spinach leaves according to a method described (7); a slight modification was made by replacing phenothiazine Affigel with Phenyl-Sepharose CL-4B. The purity of the protein was judged by the characteristic ultraviolet absorption spectrum and the absence of tryptophan fluorescence. Moreover, following electrophoresis on sodium dodecyl sulfate gels, only one band was observed (7).

Labeling of spinach calmodulin

Freshly isolated calmodulin was labelled with a fluorescence probe, thiolyte MQ. This probe is highly reactive with reduced thiol groups. The probe concentration was evaluated by measuring its absorbance (ε_{378} = 5700 M cm^{-1}) (9). The protein concentration was determined by the Bradford assay (10). The molar ratio of [label]/[calmodulin] was found to be unity (7).

Fluorescence studies of labelled calmodulin

Fluorescence experiments were performed on an SLM spectrofluorimeter, model 4800, (Urbana, IL), which was interfaced with a Hewlett-Packard desk top computer, HP-85, and a plotter to aid in data acquisition and analysis. The steady-state fluorescence anisotropy, r, was measured by using the T-format method. Two Corning filters, CS-72, were placed in the emission pathways. The excitation wavelenth was at 385 nm. Fluorescence lifetimes were determined according to the phase shift and demodulation methods (11).

2.4. Analysis of the data

The Y-function is dependent on the thermal coefficient of frictional resistance, b(T), to rotations of the fluorophore [6]. If a fraction, f_1, of the protein molecules is in substate 1, while the other fraction, f_2, occupies substate 2, the corresponding thermal coefficients are b_1 and b_2, respectively. The ratio, f_2/f_1 = K, is the equilibrium constant, and

$$b(T) = b_1 f_1 + b_2 f_2 \qquad (1)$$

The Y-function is defined as

$$Y = \ln(\eta_o/\eta) = b(T) \times (T - T_o)$$
$$= \ln\{[r(0)/r] - 1\} - \ln(RT\tau/V\eta_o) \qquad (2)$$

where η and η_o are viscosities at temperature T and a reference temperature, T_o (293.1°K), respectively. b is the thermal resistance coefficient. r is the anisotropy measured at temperature, T, r(0) is the apparent limiting anisotropy (11). For the bimane-labelled protein, we obtained a value of r(0) = 0.2142 (see Chapter II) from a Perrin plot (7). V is the molecular volume of the fluorophore probe, calculated from V=Mr(g/M)/density(g/cm^3)=146.6. τ is the fluorescence lifetime measured, and R is the gas constant. Since the Y-function is the logarithm of a ratio of viscosities, a low value of the Y-function is associated with restricted fluorophore rotation because of a high viscosity.

The standard enthalpy at the critical temperature T_c can be derived from

$$\Delta H = [(dY/dT)_{T_c} - 0.5(b_1+b_2)][4RT_c^2/t_c(b_2-b_1)] \qquad (3)$$

T_c is the critical temperature, where K = 1, and $t_c = T_c - T_o$. The critical temperature was obtained from a linear regression analysis

of the data points. The slope at this temperature was determined after fitting the experimental data to theoretical curves using a non-linear regression scheme (Marquardt's algorithm) on an IBM-XT computer. The correlation coefficient for these kinds of statistical analyses was better than 0.98.

Results

Fluorescence anisotropy and lifetime studies were performed on bimane-labelled spinach calmodulin in Mops buffer over the temperature range 10 - 40°C. From these data corresponding Y-function values (Fig. 4.1) were calculated using eq. 2.

Upon binding of the first two Ca^{2+} ions to spinach calmodulin, the Y-function values increased compared with corresponding values of apocalmodulin, at all temperatures tested (Fig. 4.1). Following binding of the first two Ca^{2+} ions to calmodulin's high affinity binding sites III and IV (1), a perturbational signal was locally elicited, producing conformational changes which, in turn, can be monitored at the bimane-labelled region I. Relative to apocalmodulin, the probe's rotational motions became apparently less restricted in $(Ca^{2+})_2$-calmodulin. In the presence of saturating Ca^{2+} concentrations, where Ca^{2+} also occupies region I, the Y-function values were higher at temperatures below 298°K, while those above that temperature were lower, compared with corresponding values derived from apocalmodulin (Fig. 4.1). Below 298°K and in the presence of saturating Ca^{2+}

concentrations, the probe therefore experienced less rotational constraint compared with that above this critical temperature. This temperature dependence is probably a manifestation of conformational changes occurring at the critical temperature of 298°K. At a molar ratio of [Al]/[calmodulin] = 3:1, addition of aluminum produced a change which, in turn, restricted probe rotation more than that observed in apocalmodulin above 293°K. Below this temperature the rotational characteristics were reversed (Fig. 4.1).

The thermal resistance coefficients, b_1 and b_2, are listed in Table 4.1. In the low temperature range, the values of the slopes, b_1, were larger than those in the higher temperature range, b_2. All b_1 and b_2 values decreased as the Ca^{2+} concentration of the solution increased.

Concerning changes of the thermal resistance coefficient (Table 4.1), it appeared that the effective resistance to rotation experienced by the fluorophore comprises two temperature-dependent contributions, viz., the protein conformations and the solvent viscosity. It is known that Ca^{2+}- and Al-binding to calmodulin lead to pronounced conformational changes in the protein (1, 7, 12, 13). Ligand-triggered changes are also reflected in the enthalpy values (Table 4.1) observed at the critical temperature, where the protein was being transformed from substate 1 into substate 2. For apocalmodulin this enthalpy change was 8.0 kcal $mol^{-1}K^{-1}$, while for $(Ca^{2+})_2$-calmodulin the enthalpy value decreased to 2.9 kcal $mol^{-1}K^{-1}$. The probe residing in region I seemingly became more sensitive to temperature changes following binding of two Ca^{2+} ions at regions III and IV. Structural

Fig. 4.1. Y(T) vs. temperature for bimane-labelled spinach calmodulin . Fluorescence studies were performed on an SLM spectrofluorimeter, model 4800. The labelled calmodulin was dissolved in Mops buffer (10 mM, pH 6.5, 0.1 M KCl) to reach a concentration of 7 μM. (●), apocalmodulin; (○), calmodulin + $2Ca^{2+}$; (■), calmodulin + $5Ca^{2+}$; and (▲), calmodulin + $5Ca^{2+}$ + 3Al.

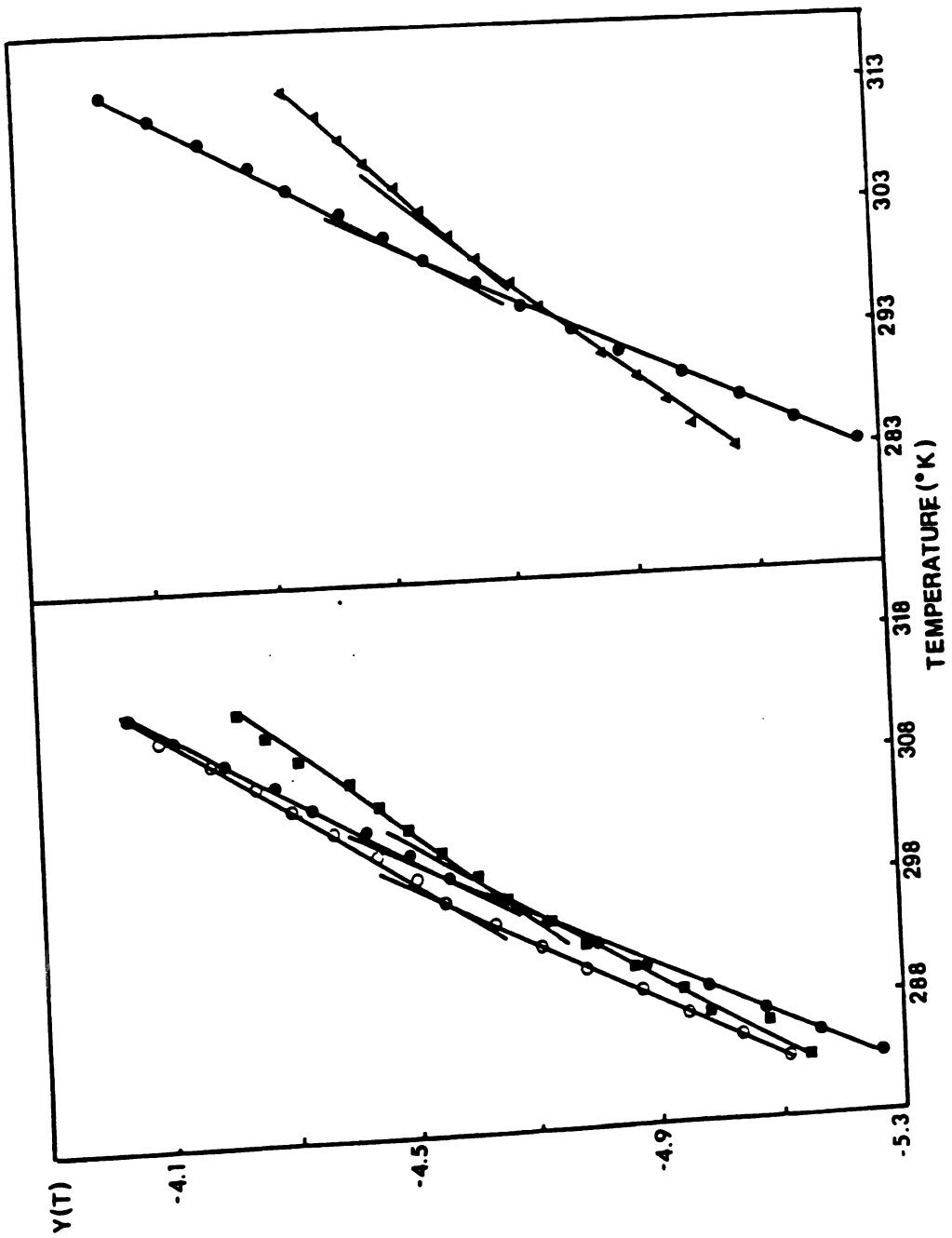
Y(T)
-4.1
-4.5
-4.9
-5.3
288
298
308
318
283
293
303
313
TEMPERATURE (°K)

Table 4.1. Physical parameters* for spinach calmodulin in the presence of ligands

calmodulin	b_1	b_2	b_1/b_2	T_c	Δ H	Δ S
apocalmodulin	4.7	3.4	1.4	299	8.0	26.8
CaM + $2Ca^{2+}$	4.2	3.0	1.4	297	2.9	9.8
CaM + $5Ca^{2+}$	3.7	2.5	1.5	298	5.3	17.8
CaM + $5Ca^{2+}$ +3Al	2.7	2.2	1.2	299	8.2	27.4

* b_1 and b_2 are listed as percent decrease per degree, T_c is the critical temperature in ^{o}K with an error of $\pm$ $1^{o}K$, Δ H in kcal per mol, Δ S in kcal per mol per degree.

perturbations at these latter sites produced conformational changes which could be monitored at the defined site of the fluorophore.

Following further addition of Ca^{2+} until saturation, one of the Ca^{2+} ions binds to Ca^{2+} binding region I. Consequently new bonds are established which render the probe's rotational motions less prone to temperature changes, as indicated by $\Delta H = 5.3$ kcal $mol^{-1}K^{-1}$. When aluminum (3:1) is present, the probe's motions appear to be less disposed to temperature changes as indicated by $\Delta H = 8.2$ kcal mol^{-1} K^{-1}. These Al-related changes of motions may be a consequence of conformational changes monitored at the probe's residing site.

Discussion

Regarding calmodulin's multifunctional roles (1), the inherent flexibility of the polypeptide chain, in the presence and absence of Ca^{2+}, is apparently of great import (3). This flexibility is in part determined by rotational barriers generated by bonds such as those of peptide linkages and hydrogen bonds in helix structures. Like that of some other proteins, calmodulin's flexible structure rapidly fluctuates around its equilibrium configuration in solution. Associated local motions are generally thought to be crucial for biochemical activities (4, 5). Molecular dynamics analysis of proteins indicated that there exist a multitude of conformational substates which are accessible by rapid structural fluctuations of the protein (14). When triggered locally by the first two Ca^{2+} ions, bound to the carboxyl terminus

lobe of the calmodulin dumbbell (15), conformational changes can be monitored through the strategically anchored fluorophore. These may originate from changes of segmental motions and/or global protein motions which, in turn, are produced by ligand-triggered perturbations of local protein structure. Therefore, these types of perturbations become manifest in changes of physical properties of fluorophore rotation.

We have also shown that spinach calmodulin, both in its apoform and in presence of metals, undergoes a substate change at a temperature of 25°C. This temperature value is significantly below the major thermal unfolding of the protein, viz., at 55°C for apocalmodulin and at > 90°C for calmodulin in the presence of Ca^{2+} (16). In biochemically active calmodulin, i.e., $(Ca^{2+})_4$-calmodulin generated in the presence of saturating Ca^{2+} (Table 4.1), the enthalpies lie below those of calmodulin which is biochemically inactive (apocalmodulin) or whose regulatory capacity has been greatly diminished by aluminum ions (13). Furthermore, in $(Ca^{2+})_4$-calmodulin the lower enthalpy seems to reflect the sensitive dynamic aspects of calmodulin, essential for the transfer of information within the macromolecule in response to regulatory stimuli. Again in biochemically active calmodulin, the Ca^{2+}-protein interaction characteristics probably remain intact (17) in both structures associated with the substates below and above the critical temperature. The temperature-dependent changes observed in apocalmodulin versus $(Ca^{2+})_4$-calmodulin (Fig. 4.1) may be related in part to their respective degree of hydration. This is illustrated, for example, in studies indicating that water molecules are removed at

each coordination site of Ca^{2+} (18). Furthermore, in biochemically active calmodulin a Ca^{2+}-induced exposure of hydrophobic surface was observed (19). Similarly, concomitant with the breakage of hydrogen bonds (13), changes in solvation structure are to be expected upon application of aluminum ions to $(Ca^{2+})_4$-calmodulin.

In summary, thanks to its finely tuned, Ca^{2+}-dependent flexibility, calmodulin is capabable of fulfilling regulatory functions.

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LIST OF REFERENCES

1. Klee, C.B., and Vanaman, T. C., (1982) Calmodulin. Adv. Protein Chem. 35: 213-321.

2. O'Neil, K.T., Wolfe, H.R., Erickson-Vittanen, S., and DeGrado, W.F., (1987) Fluorescence properties of calmodulin-binding peptides reflect alfa-helical periodicity. Science 236: 1454-1456.

3. Lambooy, P.K., Steiner, R.F., and Sternberg, H., (1982) Molecular dynamics of calmodulin as monitored by fluorescence anisotropy. Arch. Biochem. Biophys. 217: 517-528.

4. Karplus, M., and McCammon, J. A., (1981) The internal dynamics of globular proteins. CRC Crit. Rev. Biochem. 9: 293-349.

5. Careri, G., (1984) Order and Disorder in Matter. The Benjamin/ Cummings Publ., Menlo Park, CA.

6. Scarlata, S., Rholam, M., and Weber, G., (1984) Frictional resistance to local rotations of aromatic fluorophores in some small peptides. Biochemistry 23: 6789-6792.

7. Yuan, S., and Haug, A.,(1988) Ligand-triggered conformational perturbations elicit changes at the single cysteinyl residue of spinach calmodulin. Eur. J. Biochem. 175: 119-124.

8. Vanaman, T.C.,(1983) Chemical approaches to the calmodulin system. Methods Enzymol. 102: 296-310.

9. Kosower,N.S., Kosower, E.M., Newton, G.L., and Raney, H.M., (1979) Bimane fluorescent labels: labeling of normal hyman red cells under physiological conditions. Proc. Natl. Acad. Sci. (U.S.) 76: 3382-3386.

10. Bradford, M.M., (1976) A rapid and sensitive method for the quatitation of microgram quantities of protein ulitizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

11. Lakowicz, J.R., (1984) Principles of Fluorescence Spectroscopy, Plenum Press, New York.

12. Weis, C., and Haug, A., (1987) Aluminum-induced conformational changes in calmodulin alter the dynamics of interaction with melittin. Arch. Biochem. Biophys. 254: 304-312.

13. Siegel, N., and Haug, A., (1983) Aluminum interaction with calmodulin: evidence for altered structure and function from optical and enzymatic studies. Biochim. Biophys. Acta 744: 36-45.

14. Elber, R., and Karplus, M., (1987) Multiple conformational states

of proteins: a molecular dynamics analysis of myogolobin. Science 235: 318-321.

15. Babu, Y.S., Sack, J.S., Greenhough, T.J., Bugg, C.E., Means, A.R., and Cook, W.J., (1985) Three-dimensional structure of calmodulin. Nature 315: 37-40.

16. Martin, S.R.,and Bayley, P.M.,(1986) The effects of Ca^{2+} and Cd^{2+} on the secondary and tertiary strcture of bovine testis calmodulin. Biochem. J. 238: 485-490.

17. Permyakov, E.A., Shnyrov, V.L., Kalinichenko, L.P., and Orlov, N.Y., (1985) Effects of cation binding on the termal transitions in calmodulin. Biochim. Biophys. Acta 830: 288-295.

18. Mulqueen, P., Tingey, J.M., and Horrocks, W.DeW., (1985) Characterization of lanthanide (III) ion binding to calmodulin using luminescence spectroscopy. Biochemistry 24: 6639-6645.

19. LaPorte, D.C., Wierman, B.M., and Storm, D.R., (1980) Calcium-induced exposure of a hydrophobic surface on calmodulin. Biochemistry 19: 3814-3819.

CHAPTER V

SUMMARY

Shixing Yuan

The data presented in this dissertation indicate that aluminum can drastically change the conformation of the calmodulin molecule, and this, in turn, can interfere with the interactions of the molecule and its target enzymes. These results may imply that calmodulin is a potential target for deleterious aluminum ions and the aluminum intoxication may result from, at least partly, from this disturbance. We demonstrated

(a) When calmodulin interacts with specific ligands, such as cations, peptides, enzymes, molecular modifications of the interfacial area between the protein and its ligand can trigger changes in whole molecular motion, which can be monitored through a specific probe attached to the single cysteinyl residue of spinach calmodulin. In addition, this probe can also sense microenvironmental changes in response to ligands bound distant from the probe location binding. These latter changes are somehow elicited at the probe's site following transmission of information generated by ligand binding, e.g., mastoparan, to the protein (Fig. 5.1). Studies on ligand-related processes of structural changes of calmodulin appear to provide means to demonstrate deleterious effects of aluminum ions on the dynamic structure of the regulatory protein.

(b) When aluminum ions bind to calmodulin at a molar ratio of [Al]: [calmodulin] = 3:1, the whole molecular rotational motion becomes slower. This slowdown may result from the less compact structure of Al-altered calmodulin compared with that of Ca^{2+}_4-calmodulin (Fig. 5.2). In a looser molecular structure more surface area, as evidenced by an enhanced hydrophobicity, is

Fig. 5.1. An illustration of microenvironmental changes taking place in the vicinity around the EPR probe attached at Cys-26.

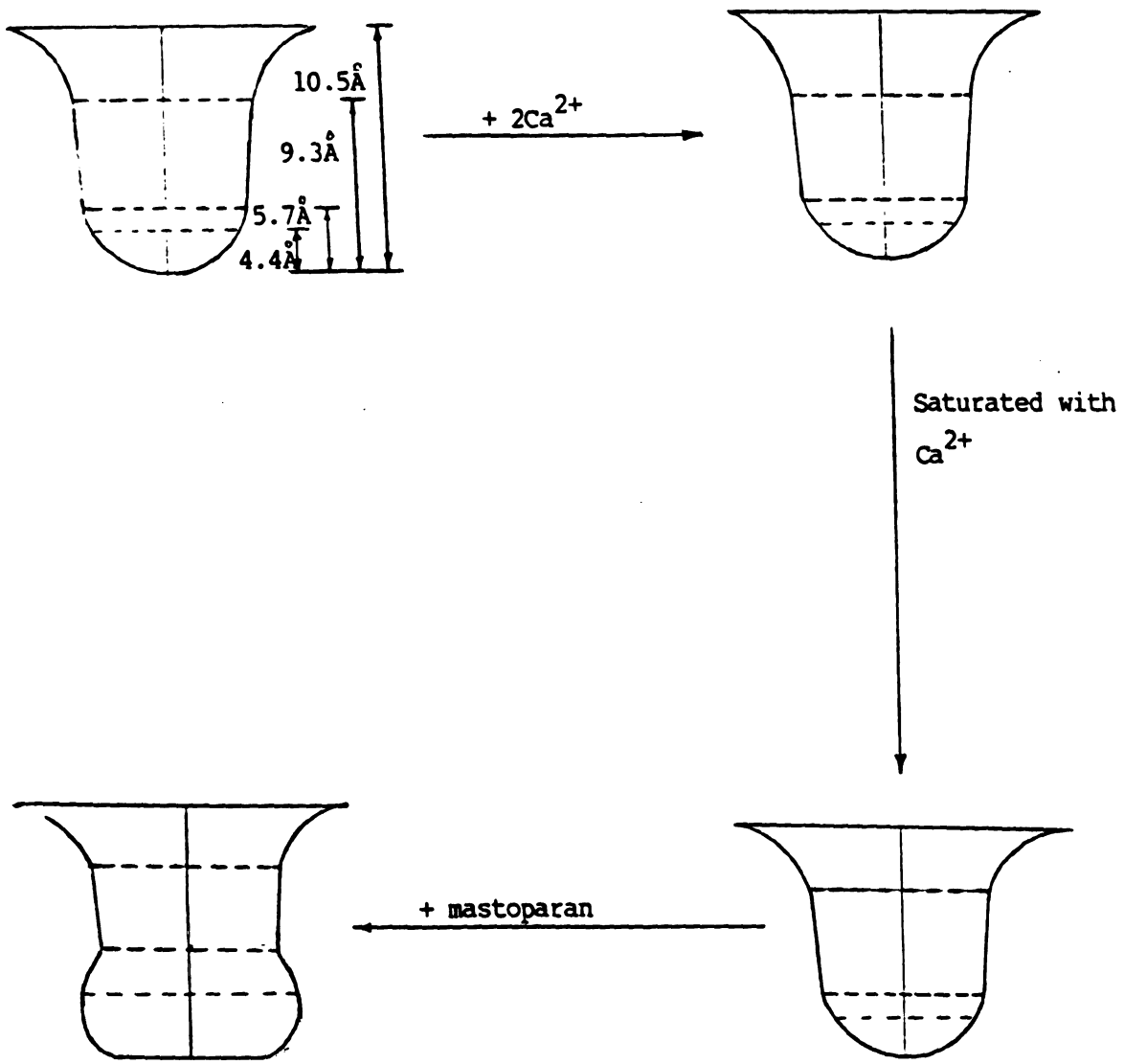
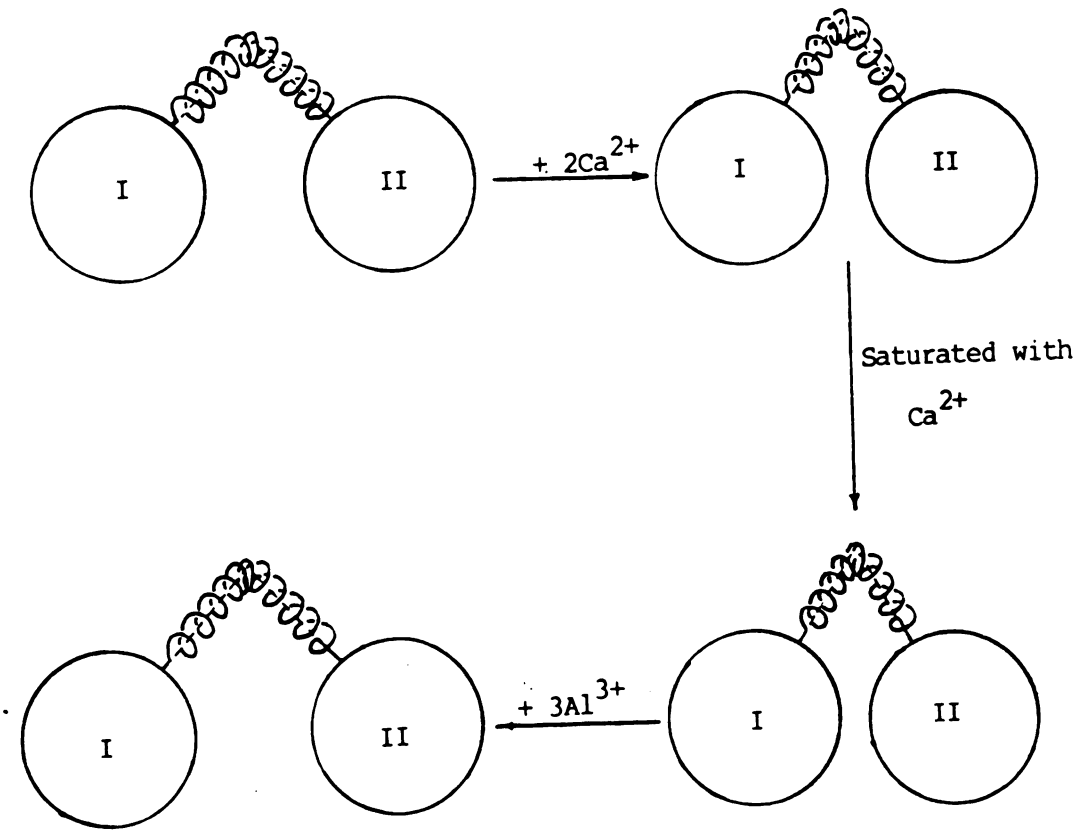

10.5Å
9.3Å
5.7Å
4.4Å
+ 2Ca2+
Saturated with
Ca2+
+ mastoparan

Fig. 5.2. An illustration of the whole molecular conformational changes of spinach calmodulin in response to ligand binding.


I
II
+ 2Ca2+
I
II
Saturated with
Ca2+
I
II
+ 3Al3+
I
II

exposed, and more friction is encountered by molecular rotations. An Al-triggered enhancement of the protein's hydrophobic area is expected to disturb the normal interaction of the molecule with its target enzymes or peptides.

(c) The temperature-controlled interchange of molecular substates may be the result of breakage or reunion of some hydrogen bonds. These events can be critical for the molecule to sense the outside environment. Following binding of aluminum ions to the protein, temperature control becomes less sensitive, more energy is needed for the interchange. In such an Al-altered protein conformation -- similar to denaturation -- it seems to be more difficult for the molecule to adjust itself to environmental fluctuations.

Taken together, aluminum may exert its toxicity by interacting with the calmodulin molecule. By changing the molecule's conformation, aluminum ions can disturb whole calmodulin-related regulatory processes. Since Ca is so involved in the regulatory processes, (including phosphatidyl innositol pathway and calmodulin-related processes) this disturbance can probably interfere with the calcium hemostasis and cause some serious toxic problems in living organisms.

APPENDIX

THE OCCURRENCE AND CORRELATION BETWEEN CALMODULIN IN RAINBOW TROUT MUCUS AND LEVELS OF pH, Ca AND Al

T.E. Lewis, S. Yuan

A. Haug and D.R. Mount

An experiment was performed to determine whether calmodulin, a calcium-regulating protein, may be used as an indicator for biologically active aluminum in lakes impacted by acidic deposition. Calmodulin has been shown to selectively bind inorganic monomeric Al. The presence of calmodulin in fish gills and mucus has been documented. The binding of Al to calmodulin causes a change in the conformation of the protein, rendering it ineffective in mediating many of the normal enzyme activities which require calcium. Rainbow trout were exposed to chronic, low-levels of Al for 147 days. A significant correlation was found between the amount of calmodulin in the mucus and aluminum concentrations in the test solutions. Calcium levels did not affect calmodulin levels. The use of calmodulin as an indicator of biologically relevant Al in surface waters is proposed.

Introduction

As a result of increasing acidification in various watersheds elevated levels of aluminum have been observed in soil and surface water (1, 2, 3, 4). In both field and controlled laboratory experiments aluminum has been shown to be highly toxic to fish (5, 6, 7, 8, 9). The toxicity of Al to fish has been shown to be positively correlated with the concentration of inorganic monomeric Al (5, 6, 10). The exact mechanism(s) of Al toxicity is not fully understood. It appears that the gill is the target organ for the toxic action of Al. Of the organs analyzed in smallmouth bass (Micropterus dolomieui) exposed to high Al levels, the gill filaments had the highest concentration of Al (11). Visible symptoms of Al toxicity include coughing response, hyperventilation, and excessive mucus clogging of the gills (10). Accompanying these visible symptoms are more specific responses such as rapid loss of sodium and chloride from plasma and lowered blood oxygen tension (10). At the cellular level the toxic mode of action remains elusive. Cleveland et al. (12) observed a reduction in RNA synthesis and RNA/DNA ratios in fish exposed to pH 5.5 in the presence of Al. This observation may be indicative of disruption or deactivation of metabolic pathways. Reduced carbonic anhydrase and Na^+-K^+-ATPase activity in gills of salmonids exposed to Al-containing waters has been reported (13).

Recently, the presence of calmodulin (CaM), a calcium-regulating protein, has been reported in fish gills and mucus (14, 15). Calmodulin selectively binds inorganic monomeric Al causing conformational changes

in the protein (16,17). Aluminum-induced conformational changes cause a reduction in the ability of calmodulin to mediate Ca-dependent phosphodiesterase and ATPase activity (16,18). Calmodulin also plays a key role in coordinating the effects of secondary messenger systems in response to cellular stimulation (19). Given the involvement of calmodulin in numerous biochemical pathways, its interaction with aluminum may be a key lesion in the broadly defined syndrome of aluminum toxicity (16).

The present study was undertaken to establish a relationship between Al concentration in aqueous solution and the presence and activity of CaM in the mucus of adult rainbow trout. Fish were exposed to various levels of pH, Ca, and Al. Mucus was collected and the amount of CaM was determined. The ability of the Al-exposed CaM to activate the phosphodiesterase enzyme system was also evaluated.

Materials and Methods

Exposure Conditions

Test water was artificial soft water, reconstituted from deionized water (U.S. EPA, 1979). Well water was treated sequentially by iron oxidation/filtration, acidification (to break carbonate complexes), reverse-osmosis, forced-draft deaeration and deionization. This water was delivered to a 700 L fiberglass headtank, where well water and NaCl solution were added to a Ca concentration of 0.5 mg/L and a Na

concentration of 1.3 mg/L. An automatic titration unit (Leeds and Northrup Model) maintained this water at pH 6.5 by addition of dilute sulfuric acid or potassium hydroxide. Two 1 horsepower water chillers (Frigid Units, Inc.) thoroughly mixed and aerated the water. After reconstitution, water was delivered to each of three 180 L fiberglass headtanks, one for each experimental pH. Here, the water was adjusted to nominal pH by automatic titrator using dilute acid, and was thoroughly mixed with a recirculating pump (5 gpm, March, Inc.). Eight combinations of pH, Ca, and Al were achieved addition of Ca and Al stock solutions (both as chlorides) from Marriotte bottles into continuous-flow serial dilution systems, Test chambers were 700 L round fiberglass tanks, each receiving 5.5 L/min. of test water (11.3 volume additions/day). Each chamber received moderate aeration. The test temperature was maintained at $9^{o}C$.

Test Organism

Test fish were 2-1/2 yr. old rainbow trout (Salmo gairdneri) (Belaire strain) obtained from the Colorado State Fish Hatchery in Crystal River, CO on 15 April, 1986. Shortly after arrival at the University of Wyoming laboratory, some fish began to show signs of "caudal peduncle" or "coldwater" disease (Cytophaga psychrophilia). Treatment with antibiotic food (Romet 30) was initiated. Visibly affected fish were destroyed. The remaining fish responded favorably to treatment and were held without recurrence of the disease. Fish were arbitrarily assigned to the eight exposure chambers (18 fish/chamber).

From 3 to 9 July, the culture water was gradually changed from well water to artificial soft water (pH 6.5, 2 mg/L Ca, 1.3 mg/L Na) and acclimation to this water continued until August. Over 11 to 18 August, pH and Ca concentration in all chambers was gradually reduced to nominal. Aluminum addition began on 18 August; this was designated as day 0 of exposure. Mean weight at the start of exposure was 1089 g.

Mortality, pH, temperature, and stock solution delivery were monitored daily. dissolved oxygen and ammonia were checked periodically and maintained within acceptable limits. Floating trout chow (Purina) was fed once daily at 0.8% body weight/day, and all tanks were cleaned daily. One to two times per week, samples were taken from all tanks for chemical analyses. Cations (Al, Ca, Na, Mg, K) were measured by atomic absorption spectrometry (Perkin-Elmer) using flame or graphite furnace techniques Standard methods (1982) protocols were employed. Aluminum was fractionated into total, reactive, and inorganic forms after the method of LaZerte (20). Anions (Cl, F, NO_3^{2-}, SO_3^{2-}) were determined by ion chromatography.

Sample Collection

Mucus samples were collected on day 147 of exposure (12 January 1987), after the method of Flik et al. (15). Fish were gently netted from the tank and anesthetized with MS-222 (Sigma Chemical Co.) in ambient tank water which was titrated back to nominal pH with KOH. Once anesthetized, each fish was grasped by the lower jaw and held over a 3000 mL beaker. Then the body surface was rinsed with 30 to 50 mL of

buffer solution. The buffer solution consisted of 0.0137 M Tris, 0.12 M NaCl, and 0.003 M KCl (pH 7.4). Samples were transferred to polypropylene centrifuge bottles, chilled to 4°C, and shipped on ice to Michigan State University for CaM determinations.

Calmodulin Assays

A calmodulin [^{125}I]-radioimmunoassay kit (New England Nuclear Co., Boston, MA) was used for CaM quantitation in fish mucus samples. Phosphotase and activator-free 3':5'-cyclic nucleotide-phosphodiesterase were obtained from Sigma Chemical Co. (St. Louis, MO). Protein assay reagents were purchased from Bio-Rad Laboratories (Richmond, CA). All other chemicals were of the highest quality available.

Mucus samples in solution were centrifuged at 1000 x g for 10 min at 4°C. The protein content of the mucus in the centrifugate was measured according to Bradford (21). Bovine gamma globulin was used as a standard material. Stimulation of cyclic AMP-dependent phosphodiesterase activity by CaM was determined by a one-step procedure (22) involving two enzymes, viz., phosphodiesterase and alkaline phosphotase incubated together to liberate inorganic phosphate in a single step. The phosphate released by the enzyme was quantitated by the Malachite green method (23). The centrifugate was then incubated at 100°C for 5 min. Calmodulin content in the incubated sample was determined as outlined above. Preheated bovine brain CaM served as a standard. Calcium-binding proteins such as troponin C and parvalbumin have been shown not to react in the calmodulin assay procedure.

Statistical Analysis

A balanced analysis of variance was performed on the data to determine whether there was a significant relationship between the amount of CaM and levels of pH, Ca, and Al. An ANOVA procedure found in the statistical software package Statgraf was employed for this purpose. The data were separated into three subsets for ANOVA analysis. The first subset consisted of two levels of Ca with Al and pH fixed. The second subset consisted of three pH levels with Ca and Al fixed. The final subset had two levels of Al with Ca and pH fixed. Due to small experimental design not enough degrees of freedom were available to assess component interactions.

Results

Table A.1 summarizes the results of the experiment. There are some general trends apparent in the data. The CaM content per mg of total protein in mucus collected from fish exposed to aluminum stress is generally greater than that from nonstressed fish. In mucus derived from Al-stressed fish, the total protein concentration was found to be similar to that of mucus from nonstressed fish. In addition, there appears to be a pH dependence of the average CaM content, as illustrated by the RIA values at pH 6.5 compared to negligible values obtained at pH 4.8 and 5.2. These trends were tested statistically. The results of ANOVA procedures are presented in Table A.2. The effects of

Table A.1. Results of CaM [^{125}I]-radioimmunoassay performed on mucus collected from rainbow trout held a various pH, Ca, and Al levels for 147 days.

sample	pH (units)	Ca (mg/L)	Al (µg/L)	CaM (µg/g)	phosphodiesterase Pi release (µM/mg.hr)
effects of Ca at fixed pH and Al					
1	5.2	2	30	n.d.	n.d.
2	5.2	2	30	1.65	12.2
3	5.2	2	30	n.d.	n.d.
4	5.2	2	30	0.43	7.1
5	5.2	4	30	0.67	3.9
6	5.2	4	30	0.68	5.8
7	5.2	4	30	1.14	4.3
8	5.2	4	30	0.61	n.d.
effects of pH at fixed Ca and Al					
10	4.8	2	0	n.d.	n.d.
11	4.8	2	0	n.d.	n.d.
12	4.8	2	0	n.d.	n.d.
13	4.8	2	0	n.d.	n.d.
14	4.8	2	0	0.36	n.d.
15	5.2	2	0	n.d.	n.d.
16	5.2	2	0	n.d.	n.d.
17	5.2	2	0	n.d.	n.d.
18	5.2	2	0	n.d.	n.d.
19	6.5	2	0	0.30	2.3
20	6.5	2	0	0.11	0.5
21	6.5	2	0	0.42	0.4
22	6.5	2	0	0.13	0.4
23	6.5	2	0	n.d.	0.9
effects of Al at fixed pH and Ca					
1	5.2	2	30	n.d.	n.d.
2	5.2	2	30	1.65	12.2
3	5.2	2	30	n.d.	n.d.
4	5.2	2	30	0.43	7.1
16	5.2	2	0	n.d.	n.d.
17	5.2	2	0	n.d.	n.d.
18	5.2	2	0	n.d.	n.d.
19	5.2	2	0	n.d.	n.d.

Note: n.d. = not detectable

Table A.2. Results of ANOVA procedure to test for relationship between Ca, pH and Al and the amount of CaM in fish mucus.

response variable: CaM content in fish mucus					
source of variation	sum of squares	degrees of freedom	mean square	F-ratio	probability (>F)
total	2.13635	7			
Ca	0.13005	1	0.13005	0.389	0.56221
error	2.00630	6	0.33438		
total	0.30054	13			
pH	0.08578	2	0.04289	2.197	0.15749
error	0.21476	11	0.01952		

pH and Ca on CaM content in the mucus were not significant. pH had a slightly larger F statistic and probability than Ca. The Al/CaM interaction could not be determined statistically due to the lack of variance in the 0 μg Al/L treatment, i.e., no CaM was detected in any of the four replicates. This finding significant, irrespective of statistical evidence. At constant pH and Ca levels the presence of 30 μg Al/L in solution resulted in the secretion of CaM into the fish mucus. In the absence of Al at the same pH and Ca levels no CaM was detected.

The presence of CaM in fish mucus is further supported by data derived from experiments on the ability of mucus to stimulate CaM-free cyclic nucleotide phosphodiesterase activity (Table A.1). The CaM-related stimulatory effect of the mucus was concentration-dependent, and was therefore more pronounced using mucus collected from Al-stressed fish. A decrease in phosphodiesterase activity is expected when Al binds to CaM (16). However the Al:CaM complex must be intact to observe a decrease in activity. The mucus collected from a single surviving fish exposed to an Al level of 70 μg/L was analyzed for Al by graphite furnace AA. While this fish had the greatest Al exposure no Al was detected in the calmodulin fraction.

Discussion

The presence of CaM in the mucus of rainbow trout is supported by two lines of evidence. Both the radioimmunoassay determination and the

phosphodiesterase assay provided positive results for the presence of CaM in mucus. These findings support the observations made by Flik et al. (15). With aluminum absent, at a Ca concentration of 50 μM, pH 6.5, an average CaM content of 0.2 μg/mg total protein in fish mucus in the present study seems to be of the same order of magnitude as that found in tilipia mucus preparations obtained from fish held in tapwater where the Ca concentration varied from 200 to 800 μM (15). Flik and coworkers observed less CaM in mucus at lower Ca levels. At the two levels of Ca tested here no statistically significant difference was observed in CaM content.

The increased CaM content in Al-stressed fish may be due to greater total protein content in the mucus. In mucus derived from Al-stressed fish the total protein concentration was found to be similar to that of mucus from nonstressed fish. Therefore, the Al-induced enhancement of mucus CaM content does not seem to result from Al-related breakage of cells at the fish surface. The enhanced presence of calmodulin in mucus is probably a response to Al stress for the purpose of maintaining membrane integrity and regulatory processes in epithelial cells. Flik et al. (15) proposed that the presence of CaM in the mucus may related to enzymatic control of integumental permeability. Calmodulin in fish mucus may serve as an extracellular chelator of Ca. Calcium plays a major role in membrane permeability by its interaction with phospholipids. Aluminum stress in fish is manifested by ionic imbalances (10). The effects of Al on the ability of CaM to mediate enzymatic control of membrane permeability cannot be discounted as a possible lesion in overall Al toxicity. Aluminum has been reported to inhibit the

Na^+-K^+-ATPase system in fish (13). Inasmuch as CaM mediates this enzyme system, Al binding to CaM may be the primary cause for this inhibition.

The lack of discernable trends in phosphodiesterase activity was expected. The Tris component of the buffer used to rinse mucus from the fish body contains potentially active binding sites for Al. Ganrot (24) reported the tendency of Al to "migrate" from one binding site to another in biological systems. If the stability of the Al:Tris complex exceeds that of the Al:CaM complexes, then Al may redistribute to Tris binding sites. This occurrence would return CaM to its normal conformation thus restoring its ability mediate phosphodiesterase activity.

Extremely low concentrations of Al were used in the present study (0-70 μg/L). Even at these low concentrations a significant correlation between the concentration of Al in solution and the amount of CaM in the fish mucus was apparent. Much higher concentrations of Al can be expected in lakes and streams impacted by acidic deposition (>200 μg/L) (4). At elevated Al levels the increase in CaM may be more pronounced.

The presence of calmodulin in fish mucus may serve as an indicator of stress induced by biologically active Al in surface waters. Confounding the response of CaM to Al may be variations in calcium levels. Calcium has been reported to affect the concentration of CaM in fish mucus (15). However, the magnitude of increase in CaM over a given range of calcium may not be as significant as that observed for Al. The results of ANOVA procedures indicated that calcium had no significant correlation with CaM in the present study. Given the large increase in

CaM at low concentrations of Al, Ca may not act as a significant "interference" in the detection of Al-induced CaM

Conclusions

Rainbow trout exposed to chronic, low-levels of Al exhibited elevated levels of CaM in the mucus. Calcium and pH levels also appear to effect the amount of CaM in the mucus, although this effect was not significant. The results clearly indicate the relationship between Al concentration in solution and the amount of calmodulin in rainbow trout mucus. despite the extremely low Al levels used in the experiment the secretion of calmodulin into the mucus was significant. Given the involvement of CaM in membrane permeability and various enzyme systems, Al binding to this protein may be the first mode of action in Al toxicity syndrome in fish. It appears that calmodulin may serve as an indicator of stress induced by the presence of biologically relevant Al in surface waters impacted by acidic deposition.

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LIST OF REFERENCES

1. Cronan, C.S., and Schofield, C.L., (1979) Aluminum leaching response to acid precipitation: effects on high-elevation watersheds in the northeast. Science 204(20): 304-306.

2. Johnson, N.M., Driscoll, C.T., Eaton, J.S., Likens, G.E., and McDowell, W.H., (1981) "Acid rain" dissolved aluminum and chemical weathering at the Hubbard Brook Experimental Forest, New Hampshire. Geochim. Cosmochim. Acta 45: 1421-1437.

3. David, M.B., and Driscoll, C.T., (1984) Aluminum speciation and equilibria in soil solutions of a haplorthod in the Adirondack Mountains (New York, U.S.A.). Geoderma. 33: 297-318.

4. Driscoll, C.T., Baker, J.P., Bisogni, J.J.Jr., and Schofield, C.L., (1984) Aluminum speciation and eqilibria in dilute acidic surface waters of the Adirondack region of New York state. in: Geological Aspects of Acid Deposition, Acid Precipitation Series. Vol. 7, (O.P. Bricker ed.), Butterworth Publ., Stoneham, MA. pp55-75.

5. Driscoll, C.T., Baker, J.P., Bisogni, J.J.Jr., and Schofield, C.L., (1980) Aluminum speciation and its effects on fish in dilute acidic water. Nature 284: 161-163.

6. Baker, J.P., and Schofield, C.L., (1982) Aluminum toxicity to fish in acidic waters. Wat. Air Soil Pollut. 18: 289-309.

7. Baker, J.P., (1982) Effects on fish of metals associated with acidification. in: Proceedings of An International Symposium on Acidic Rain and Fishery Impacts on Northeastern North America, Ithaca, NY 2-5 August, 1981. (R.E. Johnson ed.), pp165-176.

8. Henriksen, A., Skogheim, O.K., and Rosseland, B.O., (1984) Episodic changes in pH and aluminum-speciation kill fish in a Norwegian salmon river. Vatten. 40: 255-260.

9. Grahn, O., (1981) Fish kills due to high aluminum concentrations in lake waters. in: Proceedings of the International Conference on the Ecological Impact of Acid Precipitation, March 1980, snsf Project Report, Oslo, Norway.

10. Muniz, I.P., and Leivestad, H., (1981) toxic effects of aluminum on brown trout (Salmo trutta, L.), in: Proceedings of the International Conference on the Ecological Impact of Acid Precipitation, March 1980, SNSF Project Report, Oslo, Norway.

11. Brumbaugh, W.G., and Kane, D.A., (1985) Variability of aluminum concentrations in organs and whole bodies of smallmouth bass (Micropterus dolomieui). Env. Sci. Tech. 19(9): 828-831.

12. Cleveland, L., Little, E.E., Hamilton, S.J., Buckler, D.R., and

Hunn, J.B., (1986) Interactive toxicity of aluminum and acidity to early life stages of brook trout. Trans. Am. Fish. Soc. 115: 610-620.

13. Staurnes, M., Sigholt, T., and Reite, O.B., (1984) Reduced carbonic anhydrase and Na^{+}-K^{+}-ATPase activity in gills of salmonids exposed to aluminum-containing acid water. Experientia 40: 226-227.

14. Flik, G., Wendelaar Bonga, S.E., and Fenwick, J.C., (1983) Ca^{2+}-dependent phosphatase and ATPase activities in eel gill plasma membranes -- I. Identification of Ca^{2+}-activated ATPase activities with non-specific phosphatase activities. Comp. Biochem. Physiol. 76(8): 745-754.

15. Flik G., Van Rijs, J.H., and Wendelaar Bonga, S.E., (1984) Evidence for the presence of calmodulin in fish mucus. Eur. J. Biochem. 138: 651-654.

16. Siegel, N., Coughlin, R., and Haug, A., (1983) A thermodynamic and electron paramagnetic resonance study of structural changes in calmodulin induced by aluminum binding. Biochem. Biophys. Res. Comm. 115: 512-517.

17. Suhayda, C.G., and Haug, A., (1984) Organic acids prevent aluminum-induced conformationals changes in calmodulin. Biochem. Biophys. Res. Comm. 119: 376-381.

18. Siegel, N., and Haug, A., (1983) Aluminum interaction with calmodulin: Evidence for altered structure and function from optical and enzymatic studies. Biochim. Biophys. Acta 744: 36-45.

19. Rasmussen, H., (1981) Calcium and cAMP as Synarchic Messengers. John Wiley and Sons, New York.

20. LaZerte, B.D., (1984) Forms of aqueous aluminum in acidified catchments of central Ontario: A methodological analysis. Can. J. Fish. Aquat. Sci. 41: 766-776.

21. Bradform, M.M., (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

22. Teo, T.S., Wang, T.H., and Wang, J.H., (1973) Purification and properties of the protein activator of bovine heart cyclic adenosine 3',5'-monophosphate phosphodiesterase. J. Biol. Chem. 248(2): 588-595.

23. Penney, C.L., (1976) A simple micro-assay for inorganic phosphate. Anal. Biochem. 75: 201-210.

24. Ganrot, P.O., (1986) Metabolism and possible health effects of aluminum. Environ. Health Perspec. 65: 363-441.