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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

RESULTS AND DISCUSSION

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

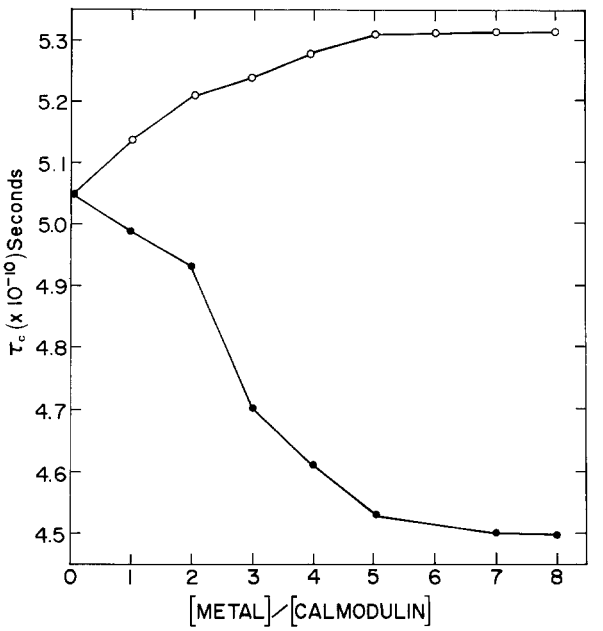

Fig. 1. Changes in spin-probe mobility of covalently labelled calmodulin due to metal addition. Values for τ_c (correlation times) were calculated as described in MATERIALS AND METHODS. Changes due to aluminum (●) or calcium (O) addition to 10 μM calmodulin at pH 6.5 are shown. Values are shown for at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

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

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

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

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

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

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

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

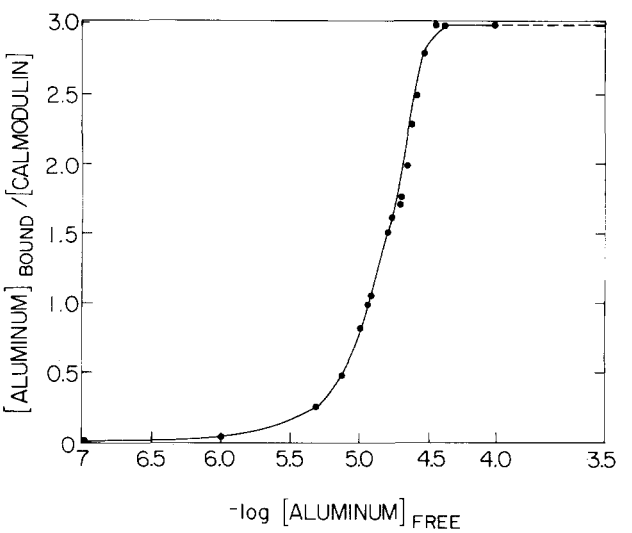

Fig. 2. Binding of aluminum to calmodulin. One-ml sample volumes of 10 μM calmodulin were dialyzed against various concentrations of aluminum chloride at pH 6.5. Aliquots were analyzed (5) for aluminum content both inside and outside of the dialysis bag after 24 h.

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

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

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

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

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REFERENCES

1. Wang, J. H. and Waisman, D. M. (1979) Curr. Top. Cell Regl. 15, 47-107
2. Klee, C. B., Crouch, T. H. and Richman, P. G. (1980) Annu. Rev. Biochem. 49, 489-515
3. Lin, Y. M. (1982) Mol. and Cell. Biochem. 45, 101-112
4. Cormier, M. J., Anderson, J. M., Charbonneau, H., Jones, H. P. and McCann, R O. (1980) in Calcium and Cell Function (Cheung, W. Y., ed.) Vol. 1, pp. 201-218, Academic Press, New York
5. Siegel, N. and Haug, A. (1983) Biochim. Biophys. Acta 744, 36-45
6. Siegel, N. and Haug, A. (1983) Physiol. Plant. in press
7. Baes, C. F. and Mesmer, R. E. (1976) in The Hydrolysis of Cations, pp. 112-113, J. Wiley and Sons, New York
8. Kilhoffer, M. C., Demaille, J. G. and Gerard, D. (1980) FEBS Lett. 116, 269-272
9. Hewgley, P. B. and Puett, D. (1980) Ann. New York Acad. Science 356, 20-31
10. Mehlhorn, R., Swanson, M., Packer, L. and Smith, P. (1980) Arch. Biochem. Biophys. 204, 471-476
11. Potter, J. D., Hsu, F. J. and Pownall, H. J. (1977) J. Biol. Chem. 252, 2452-2454
12. Hindman, J. C. and Sullivan, J. C. (1971) in Coordination Chemistry (Martell, A. E., ed.) Vol 1, pp. 393-426, Van Nostrand Reinhold Co., New York
13. Wang, C. L. A., Leavis, P. C., Harrocks, W. D. W. and Gergeley, J. (1981) Biochemistry 20, 2439-2444
14. Wang, C. L. A., Aquaron, R. R., Leavis, P. C. and Gergeley, J. (1982) Eur. J. Biochem. 124, 7-12
15. Noller, M. (1982) Acta Chim. Acad. Sci. Hung. 109, 429-448
16. Kauzmann, W. (1959) Adv. Protein Chem. 14, 1-63