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Metal-Induced Conformational Changes in Calmodulin

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Copper toxicity represents a serious problem for the aquatic environment, in particular for algae, aquatic plants, and mollusks. Copper toxicity is more pronounced in soft water than in hard water (Hartung 1973). Moreover, inhalation of copper dust and mist has been found to result in irritation of the upper respiratory tract (Proctor and Hughes 1978). The molecular basis for metal-induced alterations in cellular metabolism, in particular those caused by copper, leading to the expression of metal toxicity symptoms and potential cell death has not been elucidated. An attractive hypothesis relating to the possible mode of action of toxic metals would be that their primary target within the cell is a key regulatory protein. The potential consequences of the activation or deactivation of a regulatory protein by metals could be a cascading effect with numerous biochemical processes being affected and in turn profoundly altering cellular metabolism. One potential target for toxic metals found in all eukaryotic cells is the metal- binding protein, calmodulin, an acidic protein (M = 17 000) harboring four Ca^{2+}-binding domains. This protein has a multitude of regulatory functions [Klee and Vanaman 1982]. The regulatory activity of calmodulin is governed by the levels of free intracellular Ca^{2+} which are estimated to range from 10^{-7}M for various types of unstimulated cells to 10^{-5}M for stimulated cells.

It is our aim to report in this study on the potential of metals like copper to activate calcium-dependent calmodulin by inducing conformational changes in the protein. An attempt is made to establish a correlation between metal-induced conformational changes in the protein and physico-chemical parameters of the metal ions tested. The protein's surface hydrophobicity and alpha helix content were measured since these parameters are involved in the formation of the activated Ca^{2+}- calmodulin complex [Klee and Vanaman 1982].

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MATERIALS AND METHODS

Fluorescence and circular dichroic measurements were performed as previously described [Suhayda and Haug 1984]. The fluorescence probe 8-anilino-naphthalene sulfonic acid (ANS) was used as the sodium salt to monitor changes in the hydrophobic surface domains of calmodulin [LaPorte et al. 1980]. Calmodulin was isolated by a combination of ion-exchange and affinity chromatography techniques as previously described [Suhayda and Haug 1985]. The calmodulin-stimulated activity of the cyclic GMP-dependent nucleotide phosphodiesterase activity was measured in accordance with published techniques [Suhayda and Haug 1985].

RESULTS AND DISCUSSION

The fluorescent dye, ANS, binds to calmodulin in a Ca^{2+}-dependent manner with a K_d of 1 mM for 2.8 ANS binding sites per protein [LaPorte et al. 1980]. In the presence of Ca^{2+}, ANS prevents calmodulin binding to the phosphodiesterase suggesting that this fluorescent ligand interacts with the hydrophobic domain on calmodulin which serves as its interface with target enzymes [LaPorte et al. 1980]. Our data (Table 1) show the enhancement of ANS fluorescence of the dye-calmodulin complex in response to metal titration. The alkali cation Li^+, with an ionic radius of 0.68 A^o, did not induce a significant change in ANS fluorescence intensity when titrated to a $[Li^+]$/ [calmodulin] ratio equal to 8. This suggests that this monovalent metal cannot induce the conformational change required for the expression of the hydrophobic domain critical for the formation of the activated metal-calmodulin complex. Titration of calmodulin with Cu^{2+} under identical conditions resulted in a slight suppression in the fluorescence intensity of the ANS- calmodulin complex (Table 1). Moreover, the alpha helix content of calmodulin was slightly reduced when cations were added to the protein to a molar ratio of $[Cu^{2+}]$/ [calmodulin] equal to 8. The apparent lack of interaction between Cu^{2+} and calmodulin may in part be explained by the physico-chemical properties of this ion. Cu^{2+} has a nonhydrated ionic radius of 0.72 A^o, providing the ion with a charge-to-radius ratio of 2.78. In comparison, Ca^{2+} has an ionic radius of 0.99 A^o and a corresponding charge-to-radius value of 2.02. Possibly the smaller size of the Cu^{2+} ion in conjunction with its higher charge density, relative to that of Ca^{2+}, render it unsuitable for coordination in the Ca^{2+}-binding loops of calmodulin. Trivalent yttrium ions, Y^{3+}, titrated onto calmodulin increased the relative ANS fluorescence intensity of the dye-calmodulin complex only slightly at a molar ratio of $[Y^{3+}]$/[calmodulin] equal to 2 (Table 1). At this same ratio the alpha helix content of the protein increased slightly over that found for metal-free

Table 1. Cation-induced changes in relative ANS fluorescence intensity and alpha helix content (error 4%) of bovine brain calmodulin. F represents the fluorescence intensity of the metal-calmodulin-ANS complex while F_o is that of the metal-free calmodulin-ANS complex.

[metal] per [calmodulin] (mol/mol)	metal	relative change in fluorescence $(F/F_o) - 1$	percent in alpha helix
0	--	--	32.0
2	Li^+	+0.03	32.0
4	Li^+	+0.06	32.0
8	Li^+	+0.06	32.0
2	Cu^{2+}	-0.03	32.6
4	Cu^{2+}	-0.25	32.6
8	Cu^{2+}	-0.72	31.4
2	Y^{3+}	+0.27	33.6
4	Y^{3+}	+1.73	34.4
8	Y^{3+}	+2.57	28.2
2	Zr^4	+0.40	31.6
4	Zr^{4+}	+0.73	31.6
8	Zr^{4+}	+1.43	28.4

Note: For ANS fluorescence intensity measurements bovine brain calmodulin and the dye, ANS, were used at concentrations of 10 μM and 2 μM, respectively, at pH 6.5, in 10 mM PIPES. For the circular dichroic experiments the calmodulin concentration was 10 μM in 10 mM Tris, pH 6.5, at 22°C. The alpha helix content was derived from the measured value of the mean residue ellipticity. Each data point was replicated at least twice.

calmodulin. At a [metal]/ [calmodulin] ratio of 4, the increase in ANS fluorescence parallelled the increase in helix content of the protein. However, at the highest molar ratio used, viz. 8, the increase in fluorescence intensity was accompanied by a sharp decline in the alpha helix content of calmodulin from that found at the $[Y^{3+}]$/ [calmodulin] ratio of 4. Since the initially observed conformational changes in calmodulin upon Y ion addition resembled those induced by Ca^{2+} [Suhayda and Haug 1985], the yttrium-calmodulin complex was assayed for regulatory activity using the calmodulin-stimulated cGMP-dependent cyclic nucleotide phosphodiesterase system (Fig. 1). In this assay Y ions were added in stoichiometric amounts to metal-free calmodulin (0.2 μM) in the absence or presence of saturating Ca^{2+}-levels (50 μM) and the Y-calmodulin stimulated phosphodiesterase activity was measured at pH 7.0. The data show that the addition of 1 Y ion per calmodulin molecule results in a stimulation of phosphodiesterase

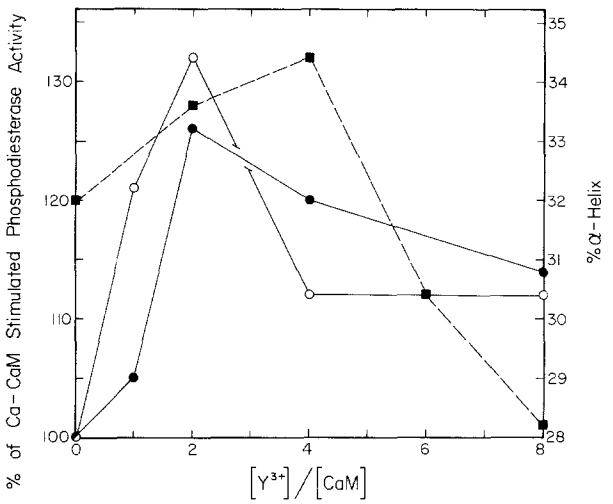

Figure.1. Stimulation of cGMP-dependent cyclic nucleotide phosphodiesterase activity by the yttrium-calmodulin complex and its relationship to the Y-induced change in alpha helix content of calmodulin (CaM). The Y-calmodulin stimulation of the enzyme was measured at pH 7.0 in the absence (O) and presence (●) of 50 μM Ca^{2+}. The concentration of the bovine brain calmodulin was 0.2 μM and Y ions were added at the respective molar ratios indicated on the abscissa. The basal activity of the enzyme measured in the absence of Ca^{2+} and calmodulin was 0.14 nmol cGMP hydrolyzed ml^{-1} min^{-1}. The 100% value corresponds to the Ca^{2+}-calmodulin stimulated maximal enzymatic activity (error about 8%) of 0.18 nmol cGMP hydrolyzed ml^{-1} min^{-1}, when the Ca^{2+} and calmodulin concentrations used were 50 μM and 0.2 μM, respectively. The Y-induced change in the helix content (■) of calmodulin was measured in Tris buffer, using a calmodulin concentration of 10 μM. Each data point was replicated at least twice.

activity which is 20% greater than that observed with Ca^{2+}-calmodulin. Increasing the metal content of the protein to 2 Y ions per calmodulin, the stimulated phosphodiesterase activity is 32% above the Ca^{2+}-calmodulin activity. However, further addition of Y ions to calmodulin, up to a molar ratio of 8, resulted in a reduction of Y- calmodulin stimulation of the phosphodiesterase to a level approximately 12% above the observed Ca^{2+}-calmodulin stimulation of the enzyme. Similar effects, although attenuated, were observed when Y ions were added to the Ca^{2+}-calmodulin complex (Fig. 1). This suggests that Y ions bind to calmodulin with a higher affinity than

Ca^{2+}. It is also apparent that neither the ANS fluorescence intensity increase nor the alpha helix content of calmodulin directly coincide with the observed changes in biochemical activity of the enzyme. It has been reported that application of Tb ions to Ca^{2+}-depleted calmodulin, at a molar ratio of 3, matched (but did not exceed) the maximal stimulation observed with the Ca^{2+}-calmodulin complex [Wallace et al. 1982]. Available data indicate that in the process of Ca^{2+}-activation the third Ca^{2+} binds to a calmodulin region corresponding to the amino acid residues 80-113 [Cox 1984]. Possibly, the first one or two Y ions applied to calmodulin interact with practically the same amino acid residue region which, in turn, leads to the formation and stabilization of the hydrophobic plate necessary for calmodulin's interaction with target proteins. It should be noted that the nonhydrated ionic radius of Y^{3+} is 0.92 A^{o}, rather similar to that of Ca^{2+} (0.99 A^{o}). In solution only the trivalent state of yttrium is stable. Commonly occurring hydrolysis products are YOH^{2+}, $Y_2(OH)_2^{4+}$, and $Y_3(OH)_5^{4+}$ [Baes and Mesmer 1976]. It is not known whether one or more of these species interact with calmodulin in solution. As to the toxicology of yttrium, it is known that yttrium compounds cause pulmonary irritation in animals. Yttrium is used in mixtures with lanthanides as phosphors in color television receivers (Proctor and Hughes 1978).

Interaction of Zr^{4+} with calmodulin, when added at a molar ratio of 8, resulted in increased fluorescence intensity of the dye-calmodulin complex and a decrease in the protein's alpha helix content (Table 1). Qualitatively similar metal- induced changes have been reported for aluminum [Suhayda and Haug 1985]. The observed Zr-effects may be related to the solution chemistry of the tetravalent cation. In dilute solution the ion exists as the following hydroxide complexes: $Zr(OH)_5^-$, $Zr(OH)_4$, and $Zr(OH)_3^+$, comprising 55%, 35%, and 5%, respectively, of the total ionic species present in solution at pH 6.5 [Baes and Mesmer 1976]. Zirconium compounds are of low toxicity, however, granulomas on human skin have been reported (Proctor and Hughes 1978). The charge-to-radius value for Zr^{4+} is 5.06, comparable to that of Al^{3+} with a value of 5.88.

In summary, there appears to be a correlation between a high charge-to-radius ratio of the metal and its ability to induce conformational changes in calmodulin that result in activation of the regulatory protein.

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