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TEMPERATURE DEPENDENT CONFORMATIONAL CHANGES IN CALMODULIN

Preeti Gangola and Harish C. Pant

Laboratory of Preclinical Studies
National Institute on Alcohol Abuse
and Alcoholism
Rockville, MD 20852

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Calcium bound calmodulin undergoes a reversible conformational change in the temperature range (22-28°C). The transition temperature depends upon the concentration of calcium bound to calmodulin. The transition occurs at higher temperature (28°C) in fully Ca^{2+} bound calmodulin as compared to those with lower Ca^{2+} concentrations (22°C). The sequence of filling the four Ca^{2+} binding sites is different for the two temperature dependent conformers.

The mechanism through which calmodulin regulates the enzymatic processes is only partially understood (1,2). It posseses four Ca^{2+} binding sites; the affinity and specificity of the sites vary considerably with the different experimental conditions (2-4). Binding of Ca^{2+} to calmodulin results in a conformational change leading to a greater helical content and more stable structure. Such a conformational change is mandatory to all enzyme activation processes regulated by CaM. This active conformer then interacts with and activates the enzyme to be regulated.

Some of the published data indicate that Ca^{2+}-CaM complex with different stoichiometries may be active toward different enzymes, adenylate cyclase activation can occur with two Ca^{2+} bound (5), whereas phosphodiesterase and myosin light chain kinase require all four sites to be filled (6-8). It is apparent that the sequential occupancy of the Ca^{2+} binding sites and accompanied conformational changes may dictate the systems regulated by CaM.

In this communication we report a temperature induced conformational change in CaM and its effect on the filling sequence of Ca^{2+} binding sites as studied by intrinsic fluorescence measurements.

Abbreviations used are: CaM, Calmodulin; EGTA, ethylene glycol bis (β- amino ethyl ether) N,N,N',N'-tetra acetic acid.

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Materials and Methods:

Calmodulin was purchased from Calbiochem Boehring Corp. and was further purified with an affinity-gel phenothiazine column purchased from Bio-Rad Laboratories. CaM was applied to the column equilibriated with 50 mM Tris-HCl (pH 7.5) containing 300 mM NaCl, 100 μM $CaCl_2$ and 1 mM 2-mercaptoethanol. The column was washed with the same buffer until the optical density of the eluant was the same as of the buffer. CaM was eluted with 5 mM EGTA, 50 mM Tris (pH 7.5) and was dialysed against double distilled water for 24 hours with 6 changes to remove EGTA carried over from the elution buffer.

Fluorescence measurements were performed with a PerkinElmer MPF-44E spectrofluorometer. Excitation was done at 277 nm; the spectral band widths were 6 nm and 8 nm for excitation and emission respectively. In order to study the temperature dependence of fluorescence, the spectra were recorded at different temperatures controlled with a Endocal temperature bath. The measurements were carried out in 20 mM Tris-HCl containing 100 mM NaCl (pH 7.5). Calmodulin concentration was 12 uM. For Ca^{2+} titrations 1 to 2 μl of a 2 mM $CaCl_2$ stock solution was added to 400 μl of CaM solution in the fluorescence cuvette and after each addition fluroescence intensity was measured at 305 nm.

Results and Discussion:

The tyrosine residues of calmodulin serve as the reporters of conformational changes induced in the molecule (4,9). CaM exhibits the characterstic fluorescence band of tyrosine at 305 nm when excited at 277 nm. Upon Ca^{2+} binding the fluorescence intensity shows a marked increase which reveals the change in the microenvironments of the two tyrosine residues as a consequence of conformational changes induced by Ca^{2+} binding. The total enhancement depends on the temperature as well as on the ionic strength of the solution. In 20 mM Tris-HCl at 25°C the fluorescence increase is only 1.8 times, whereas in the presence of 100 mM NaCl the increase is 2.5 fold. In the absence of salt, Tris-HCl causes a quenching of fluorescence, as the optical density at 277 nm does not change by addition of salt The most probable site for solvent quenching is tyrosine 138 which is located on

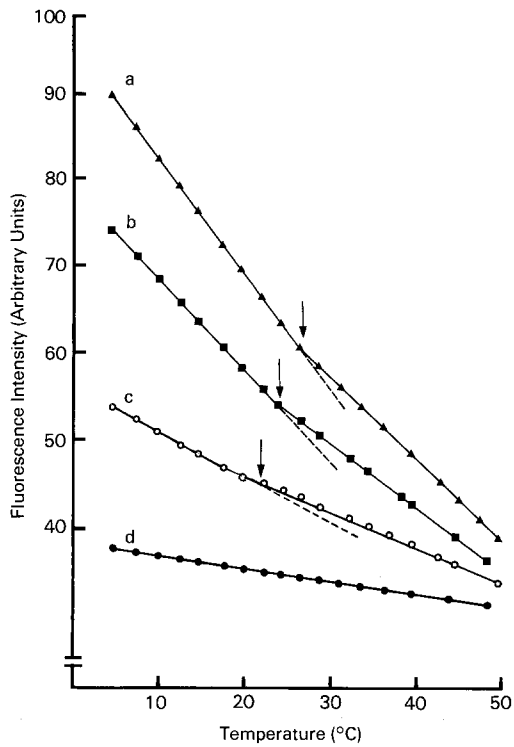

Fig 1: Temperature dependence of fluorescence intensity of CaM with added Ca^{2+} (μM); (a) 80, (b) 40, (c) 20 and (d) 0. Protein concentration is 12 μM in 20 mM Tris-HCl and 100 mM NaCl, pH 7.5.

the surface of the protein molecule and is therefore most accessible to solvent interaction.

Fig 1 depicts the temperature variation of the fluoresence intensity of CaM with variable quantities of added Ca^{2+}. As seen from curve (a) the intensity of CaM saturated with Ca^{2+}, decreases monotonically with increasing temperature in the range 5-25°C and shows the usual thermal quenching due to activation of collisions between excited tyrosine and neighbouring groups or solvent. Further heating results in a sharp deflection in the curve at 28°C. Such a change is indicative of a conformational transition affecting the structure surrounding the tyrosine residues. It is most likely that on increasing temperature the CaM molecule is unfolding and some noncovalent interactions are affected giving rise to this thermal transition and consequently a new conformer is formed. Further increase in the temperature up to 50°C again decreases the fluorescence intensity monotonically but at a slower rate and this shows the thermal quenching of the second conformer.

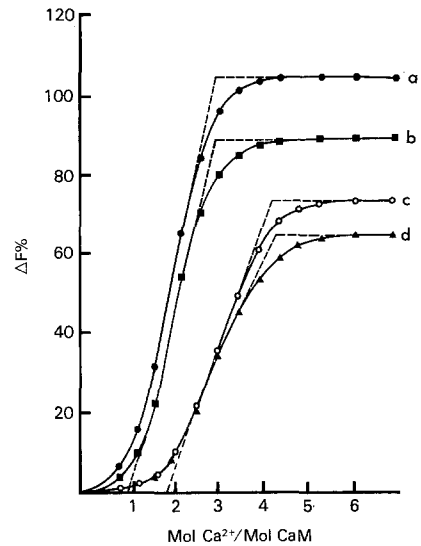

Fig 2: Titration of CaM with Ca^{2+} at temperatures (°C); (a) 10, (b) 20, (c) 26 and (d) 35. Protein concentration is 12 μM in 20 mM Tris-HCl and 100 mM NaCl, pH 7.5.

This temperature induced conformational change is observed to be reversible i.e. the spectra and intensity value can be regained in heating and cooling cycle.

Similar conformational change is observed at lower Ca^{2+} concentrations (curves b and c). As expected, for different conformers with varying Ca^{2+} concentrations, the slopes of the curves as well as the transition temperatures are different. Transition temperature shifts to lower values with decreasing Ca^{2+} concentration, viz., with 40 μM and 20 μM of added Ca^{2+} the transition occurs at 25°C and 22°C respectively. In the absence of Ca^{2+}, CaM does not show the deflection in the fluorescence intensity curve (d).

The four Ca^{2+} binding sites in CaM are designated as I - IV from the N-terminal. Among these, the two domains I and II do not contain tyrosine residues, whereas domains III and IV each has one tyrosine. We observe that the sequence of calcium binding to these four sites depends on the temperature. The filling sequence was determined as by Wallace et al (10). Fig. 2 illustrates the percent increase in fluorescence intensity as a function of Ca^{2+} concentration at different temperatures. When the tangents from the linear portions of the titration curve are drawn they intercept at the ratios of 1 and 3 moles of Ca^{2+} per CaM molecule at 20°C (curve b). There is no significant enhancement of fluorescence up to binding of 1

mol of Ca^{2+} per mole of CaM. Introduction of a second Ca^{2+} suddenly enhances the fluorescence which continues up to a ratio of 3 Ca^{2+} ions per CaM molecule before the onset of the plateau. These findings suggest the following sequence of filling of the four sites at 20°C. The first Ca^{2+} binding site filled is either domain I or II which does not have fluorescent moiety, the next two sites filled are fluorescent domains III and IV and in the last domain II or I whichever is not filled initially. Wallace et al (10) have suggested the same sequence of filling by studying the fluorescence of Tb^{3+} bound to CaM.

Similar analysis of fluorescence titration curves for different temperatures shows the temperature dependence of the filling sequence. As can be seen from fig. 2, at 10°C the Ca^{2+} binds in the same manner as at 20°C. However, at 26°C a remarkable difference is observed, namely the enhancement takes place with 3 moles of Ca^{2+} and plateau is observed at 4 moles of Ca^{2+}/CaM mole. In other words the first two Ca^{2+} go to nonfluorescent domains I and II and the last two molecules bind to domains III and IV. The same sequence is observed at 35°C (curve d). This reversal of the sequence in filling up of the site II (or I) in preference to site III, which is observed at 20°C and below, obviously must be connected with temperature induced conformational changes.

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