

Identification of Mg²⁺-Binding Sites and the Role of Mg²⁺ on Target Recognition by Calmodulin[†]

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ABSTRACT: The binding of Mg²⁺ to calmodulin (CaM) and the effect of Mg²⁺ on the binding of Ca²⁺–CaM to target peptides were examined using two-dimensional nuclear magnetic resonance and fluorescence spectroscopic techniques. We found that Mg²⁺ preferentially binds to Ca²⁺-binding sites I and IV of CaM in the absence of Ca²⁺ and that Ca²⁺-binding site III displays the lowest affinity for Mg²⁺. In contrast to the marked structural transitions induced by Ca²⁺ binding, Mg²⁺ binding causes only localized conformational changes within the four Ca²⁺-binding loops of CaM. Therefore, Mg²⁺ does not seem to be able to cause significant structural effects required for the interaction of CaM with target proteins. The presence of excess Mg²⁺ (up to 10 mM) does not change the order and cooperativity of Ca²⁺ binding to CaM, and as expected, the structure of Ca²⁺-saturated CaM is not affected by the presence of Mg²⁺. However, we found that the binding of Ca²⁺-saturated CaM to target peptides is affected by Mg²⁺ with the binding affinity decreasing as the Mg²⁺ concentration increases. Three different peptides, corresponding to the CaM binding domain of skeletal muscle myosin light-chain kinase (MLCK), CaM-dependent cyclic nucleotide phosphodiesterase (PDE), and smooth muscle caldesmon (CaD), were examined and show different reductions in their affinities toward CaM. The CaM-binding affinity of the MLCK peptide in the presence of 50 mM Mg²⁺ is approximately 40-fold lower than that seen in the absence of Mg²⁺, and a similar response was observed for the PDE peptide. The affinity of the CaD peptide for CaM also shows a Mg²⁺ dependence, though to a much lower magnitude. The Mg²⁺-dependent decrease in the affinities between CaM and its target peptides is an intrinsic property of Mg²⁺ rather than a nonspecific ionic effect, as other metal ions such as Na⁺ do not completely replicate the effect of Mg²⁺. The inhibitory effect of Mg²⁺ on the formation of complexes between CaM and its targets may contribute to the specificity of CaM in target activation in response to cellular Ca²⁺ concentration fluctuations.

The ubiquitous eukaryotic protein calmodulin (CaM)¹ is responsible for converting the intracellular Ca²⁺ signal into a wide range of physiological events. Successful signal conversion is achieved by transforming Ca²⁺-free CaM into its Ca²⁺-saturated form as the intracellular Ca²⁺ concentration rises. The Ca²⁺-saturated CaM is capable of binding to many target enzymes, including phosphodiesterase, myosin light-chain kinases, CaM kinases, and calcineurin, thereby mediating a multitude of biological events (Means et al., 1991; Vogel, 1994).

Ca²⁺-free CaM contains two globular domains connected by a flexible central linker (Zhang et al., 1995; Kuboniwa et al., 1995). Each domain contains two well-defined helix–

loop–helix EF-hand motifs that are responsible for Ca²⁺ binding. The 6 N-terminal residues in each of the four 12-residue Ca²⁺-binding sites (sites I–IV) are highly mobile (Zhang et al., 1995; Tjandra et al., 1995; M. Zhang, unpublished). Ca²⁺ binds in a sequential order to sites III and IV and then to sites I and II; a cooperative binding within each pair of the Ca²⁺ sites was observed (Andersson et al., 1983; Ikura et al., 1983; Dalgarno et al., 1984). The binding of Ca²⁺ induces large conformational changes through an orientational rearrangement of the existing secondary structural elements in the protein. The two helices in each EF-hand change their orientation from nearly antiparallel in the Ca²⁺-free state (Zhang et al., 1995; Kuboniwa et al., 1995) to almost perpendicular in the Ca²⁺-saturated state (Babu et al., 1988). Thus, both domains of CaM undergo a change from a four-helix bundle-like “closed” conformation in the Ca²⁺-free state to a well-separated “open” conformation in the Ca²⁺-saturated state (Zhang et al., 1995; Kuboniwa et al., 1995; Babu et al., 1988). The Ca²⁺-induced structural transition leads to dramatic alterations in the molecular surface of the protein. Instead of a relatively flat, hydrophilic surface characteristic of each domain of Ca²⁺-free CaM, a large solvent-exposed hydrophobic surface with a deep cavity is observed in each domain of Ca²⁺-saturated CaM (Zhang et al., 1995; Babu et al., 1988). The formation of the hydrophobic cavity is the most important structural feature

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¹ Abbreviations: Br₂BAPTA, 5,5'-dibromo-1,2-bis(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid; CaD, smooth muscle caldesmon; CaM, calmodulin; TR1C, N-terminal half-fragment (residues 1–75) of scallop calmodulin; TR2C, C-terminal half-fragment (residues 78–148) of scallop calmodulin; HSQC, heteronuclear single-quantum coherence; MLCK, skeletal muscle myosin light-chain kinase; NMR, nuclear magnetic resonance; PDE, calmodulin-dependent cyclic nucleotide phosphodiesterase.

in understanding the activation mechanism of CaM by Ca^{2+} (Ikura et al., 1992; Meador et al., 1992, 1993).

Mg^{2+} is just above Ca^{2+} in the element group IIA of the periodic table, and these two metal ions bear many similarities in their chemical properties. The intracellular free Mg^{2+} concentration is on the order of 1 mM, which is about 10^4 -fold higher than that of Ca^{2+} . Previous studies have shown that Mg^{2+} can bind to CaM with association constants ranging between 10^2 and 10^4 M^{-1} (Haiech et al., 1981; Ogawa & Tanokura, 1984; Iida & Potter, 1986; Milos et al., 1986; Tsai et al., 1987). Much controversy exists regarding the Mg^{2+} -binding sites in CaM. Microcalorimetric studies showed that there are four identical Mg^{2+} -binding sites that do not completely overlap with Ca^{2+} -binding sites in CaM (Milos et al., 1986; Tanoka & Yamada, 1993). Mg^{2+} binding studies using flow dialysis and ion selective electrode techniques, however, have shown that Mg^{2+} and Ca^{2+} share the same binding sites in both domains of the protein and that the two metal ions compete with each other (Haiech et al., 1981; Iida & Potter, 1986). $^{25}\text{Mg}^{2+}$ NMR spectroscopic studies suggested the existence of a pair of high-affinity Mg^{2+} -binding sites in the N-terminal domain of CaM (Tsai et al., 1987). Mg^{2+} binding studies using mass spectrometry detected a pair of strong Mg^{2+} -binding sites (with an association constant of 10^4 M^{-1}) in CaM that are distinct from Ca^{2+} -binding sites (Lafitte et al., 1995). Another key question which has not yet been addressed in detail is the functional relevance of Mg^{2+} to CaM's target recognition and activation, although it has been suggested that Mg^{2+} might hinder active complex formation between CaM and its targets at lower Ca^{2+} concentrations (Ohki et al., 1993). In this work, we have used two-dimensional nuclear magnetic resonance (NMR) spectroscopy to identify Mg^{2+} -binding site(s) of CaM by directly monitoring chemical shift changes of individual amino acid residues of the protein during metal ion titration. Mg^{2+} -induced structural changes have also been studied with this approach. In addition, effects of Mg^{2+} on the binding of Ca^{2+} -saturated CaM to its targets have been investigated using fluorescence spectroscopy.

MATERIALS AND METHODS

Proteins and Peptides. Uniformly ^{15}N -labeled recombinant *Xenopus laevis* CaM was expressed in and purified from *Escherichia coli* cells as previously described (Zhang et al., 1995; Ikura et al., 1990). Metal-free ^{15}N -labeled CaM was prepared following a published method (Zhang et al., 1995). Unlabeled CaM was overexpressed in and purified from *E. coli* cells as described earlier (Zhang & Vogel, 1993). Calmodulin fragments corresponding to the N- and C-terminal half-domains (residues 1–75, TR1C, and residues 78–148, TR2C) were prepared as previously described (Yazawa et al., 1980; Minowa & Yagi, 1984).

The 22-residue skeletal muscle myosin light-chain kinase (MLCK) peptide (KRRWKNFIAVSAANRFKISS), the 20-residue CaM-dependent cyclic nucleotide phosphodiesterase (PDE) peptide (QTEKMWQRLKGILRCLVKQL), and the 17-residue smooth muscle caldesmon (CaD) peptide (GVRNIKSMWEKGNVFSS) were commercially synthesized and used as described previously (Zhang et al., 1993, 1994; Zhang & Vogel, 1994). These peptides correspond to the CaM-binding domains of the respective proteins.

NMR Experiments. The titration experiments were performed using two ^{15}N -labeled CaM samples dissolved in 0.5

mL of an unbuffered 90% $\text{H}_2\text{O}/10\% \text{D}_2\text{O}$ mixture in the presence of 0.1 M KCl. The concentration of CaM was 1.0 mM. The pH of the samples was adjusted to 6.5 and maintained at this value by adding small aliquots of diluted KOH or HCl during the titration process since binding of metal ions tends to decrease the pH value of the samples. One sample was initially titrated with Mg^{2+} until 10 equiv of Mg^{2+} was added to the sample and then subjected to Ca^{2+} titration. The other Ca^{2+} - and Mg^{2+} -free sample was prepared for Ca^{2+} titration in order to allow a measure of the effect of Mg^{2+} on Ca^{2+} binding to the protein.

NMR spectra were obtained on a Varian Unity-plus 500 MHz spectrometer equipped with an actively z gradient-shielded triple-resonance probe and a pulse field gradient (PFG) driver. A PFG was used to suppress spectral artifacts and minimize the water signal (Bax & Pochapsky, 1992). Sensitivity-enhanced $^1\text{H}-^{15}\text{N}$ heteronuclear single-quantum correlation (HSQC) spectra were recorded using a published pulse sequence (Kay et al., 1992). The sample temperature was kept at 23.0 °C during collection of the FID signals. Complex FID data matrices of $512 \times 128 (f_2 \times f_1)$ were acquired for each spectrum. NMR data were processed and displayed using the software nmrPipe and nmrDraw, respectively (Delaglio et al., 1995).

Ca^{2+} Binding. Each calmodulin fragment (25 μM) was titrated with Ca^{2+} in 25 mM Tris buffer containing various concentrations of Mg^{2+} (0, 10, 50, and 100 mM), 25 μM Br_2BAPTA , and 100 mM KCl at pH 7.5. The UV absorbance of the fragment mixtures at 263 nm was monitored at room temperature on a Beckman DU-650 UV-Vis spectrometer. Ca^{2+} binding constants were determined by fitting the experimental data using the method described earlier by Linse et al. (1991a).

Fluorescence Experiments. The concentration of each stock solution of peptides was determined by the Biuret method and by UV absorption using the excitation coefficient of the single Trp residue in peptides. The CaM concentration was estimated by the Biuret method and by the UV absorption coefficient of $A_{276}^{1\%}$ (=1.8). For the binding assays between each peptide and CaM, four different concentrations of Mg^{2+} (0, 10, 50, and 100 mM) were used to study the effect of Mg^{2+} on the formation of the complexes. In another set of CaM-peptide binding experiments, the complexes containing various concentrations of Na^+ (0, 20, 100, and 200 mM) instead of Mg^{2+} were studied as a control for nonspecific ionic effects on complex formation between CaM and its targets. The concentrations of the peptides were 2.0 μM (for the MLCK peptide and the PDE peptide) and 8.0 μM (for the CaD peptide). The binding reaction was carried out in a buffer containing 25 mM Tris, 100 mM KCl, and 1 mM CaCl_2 at pH 7.5. In the case of the PDE peptide, 1 mM DTT was added to the peptide stock solution which was incubated at 37 °C for 1 h to ensure the complete reduction of the sulfhydryl group of a free Cys residue in the peptide before fluorescence measurement. The fluorescence titrations were carried out by titrating each peptide solution with an appropriate aliquot of a CaM stock solution.

Fluorescence spectra were observed at room temperature with a Perkin-Elmer LS50B fluorometer. Excitation and emission band widths were 10 and 5 nm, respectively, for the MLCK and PDE peptides. For the CaD peptide, the emission band width was adjusted to 4 nm. An excitation

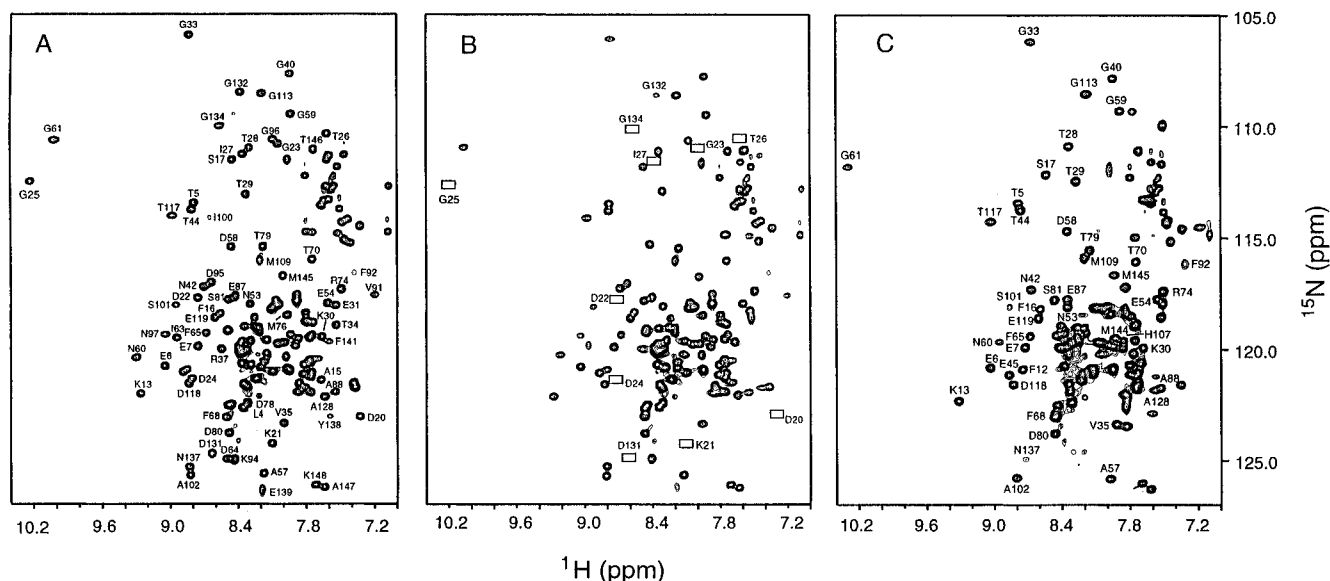


FIGURE 1: ¹H–¹⁵N HSQC spectra of uniformly ¹⁵N-labeled CaM with different concentrations of Mg²⁺ and Ca²⁺ ions. (A) Mg²⁺-free, Ca²⁺-free CaM; most of the resonances are labeled with their residue name and number. (B) CaM with no Ca²⁺ and 1 equiv of Mg²⁺; the open boxes highlight the resonances that disappear upon the addition of 1 equiv of Mg²⁺ to the sample. (C) Ca²⁺-free protein with 10 equiv of Mg²⁺; the residues that are located in the helical regions of the protein are labeled with residue numbers.

wavelength of 295 nm was selected in order to minimize contributions from the Tyr residues in CaM. Emission spectra covered wavelengths from 300 to 400 nm.

RESULTS

Mg²⁺ Ions Bind Preferentially to Ca²⁺-Binding Sites I and IV. CaM and troponin C bear close similarities with respect to their metal ion binding properties. Work on troponin C has identified a pair of specific Ca²⁺ and Mg²⁺ sites in the C-terminal domain of the protein (Potter & Gergely, 1975). Several points are still unclear: (a) the existence of specific Mg²⁺ site(s) in CaM, (b) the stoichiometry of Mg²⁺ binding to CaM, (c) the site preference of Mg²⁺ in CaM, and (d) the conformational effects on CaM of Mg²⁺ binding. In an attempt to resolve the above issues, we performed Mg²⁺ titration experiments on apo-CaM by recording ¹H–¹⁵N HSQC spectra of uniformly ¹⁵N-labeled CaM for which complete backbone assignments are available both in the Ca²⁺-free state (Zhang et al., 1995) and in the Ca²⁺-saturated state (Ikura et al., 1990).

Figure 1A shows the backbone ¹H–¹⁵N HSQC spectrum of Ca²⁺- and Mg²⁺-free CaM at pH 6.5. When the first equivalent of Mg²⁺ was added to the protein, the resonances corresponding to the first and fourth Ca²⁺-binding sites (highlighted with open boxes in Figure 1B) underwent substantial line broadening, whereas the resonances from the second and third Ca²⁺-binding sites remained almost unchanged. Such line broadening arises from the fact that the Mg²⁺-bound and the Mg²⁺-free forms of CaM were exchanging at a fast to intermediate rate on the NMR time scale. No further significant changes for the residues in sites I and IV were observed when the next 3 equiv of Mg²⁺ was added to the sample (data not shown), whereas the resonances from site II showed continuous chemical shift changes during the titration. When the concentration of Mg²⁺ reached 10 equiv, a new set of resonances were observed (Figure 1C); these resonances presumably belong to the residues from the Mg²⁺-bound Ca²⁺ sites I and IV since resonances from site II are

still undergoing chemical shift changes. At this point, we have also observed some line broadening for the resonances from site III (Figure 1C). In the course of the titration, the resonances corresponding to the helical regions throughout the protein as well as the central domain-linker region did not undergo appreciable chemical shift changes (Figure 1A–C), suggesting that Mg²⁺ binding induced conformational changes localized only to the Ca²⁺-binding loops of the protein.

Mg²⁺ Does Not Affect the Binding of Ca²⁺ to CaM. We also studied the effect of Mg²⁺ on the binding of Ca²⁺ to the protein by comparative Ca²⁺ titration studies of Mg²⁺-free, Ca²⁺-free and Mg²⁺-bound, Ca²⁺-free CaM. As expected, the first 2 equiv of Ca²⁺ bound to sites III and IV of the Mg²⁺-free sample cooperatively, and then the next two sites in the N-terminal domain were filled with Ca²⁺ (Andersson et al., 1983; Linse et al., 1991; data not shown). In the presence of 10 mM Mg²⁺, the order of Ca²⁺ binding to CaM remained the same as that in the absence of Mg²⁺, and similar cooperativity was also observed. Figure 2A shows the ¹H–¹⁵N HSQC spectrum of CaM in the presence of 1.6 equiv of Ca²⁺ and 10 mM Mg²⁺. At this point of the Ca²⁺ titration experiment, the resonances of the C-terminal residues appeared at chemical shift values corresponding to the Ca²⁺-saturated state of the protein (Figure 2A,B), whereas the resonances of the N-terminal residues still remained at the positions of the Ca²⁺-free state (Figures 1A and 2A). The Ca²⁺ titration profiles of CaM in the presence and absence of up to 10 mM Mg²⁺ were indistinguishable within experimental error, suggesting that the binding of Mg²⁺ did not change Ca²⁺ binding to the protein in an appreciable manner. However, we were unable to quantitatively measure the effect of Mg²⁺ on the dissociation constants between Ca²⁺ and CaM from the current NMR study due to experimental limitations.

Using a competitive Ca²⁺-binding dye, Br₂BAPTA, we measured the Ca²⁺ binding constants of calmodulin in the presence of various concentrations of Mg²⁺. In order to

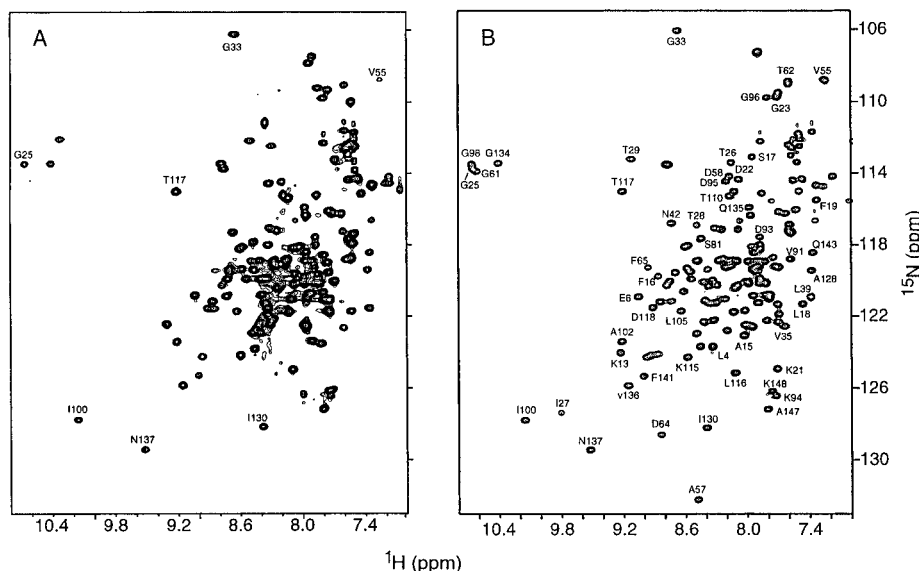


FIGURE 2: ^1H - ^{15}N HSQC spectra of CaM in the presence of 1.6 (A) and 4.5 equiv (B) of Ca^{2+} . The protein sample contains 10 mM Mg^{2+} . For clarity, only selected residues are labeled [for further details, see Ikura et al. (1990)].

Table 1: Macroscopic Ca^{2+} Binding Constants of Calmodulin Fragments in the Presence of Mg^{2+}

[Mg^{2+}] (mM)	TR1C ^a		TR2C ^a	
	log K_1	log K_2	log K_1	log K_2
0	4.7	5.7	5.0	6.0
10	4.8	5.7	4.9	5.8
50	4.5	5.3	4.6	5.6
100	4.5	5.2	4.5	5.2

^a The uncertainties are ± 0.2 for each value.

simplify the curve fitting of the experimental data, the TR1C and TR2C fragments rather than the intact protein were used to measure macroscopic Ca^{2+} binding constants of the protein, as performed earlier (Linse et al., 1991). The macroscopic binding constants of the CaM fragments are summarized in Table 1. The presence of Mg^{2+} up to 10 mM did not have a significant effect on the Ca^{2+} binding affinity and cooperativity of CaM, which is in good agreement with the results of the NMR studies described above. However, at higher Mg^{2+} concentrations, a noticeable decrease in Ca^{2+} affinity was observed for the C-terminal domain of the protein, while the N-terminal domain of the protein displayed a lower sensitivity to Mg^{2+} concentration changes. At a Mg^{2+} concentration of 100 mM, both the N-terminal and the C-terminal domains had similar Ca^{2+} binding constants.

In order to answer the question of whether the Mg^{2+} -induced decrease of the macroscopic Ca^{2+} binding constant is a result of (a) direct competition between Ca^{2+} and Mg^{2+} or (b) a pure nonspecific ionic effect, we have compared the chemical shift changes of the backbone amides induced by the addition of K^+ and Mg^{2+} . Similar to what was found in a related Ca^{2+} -binding protein calbindin $\text{D}_{9\text{K}}$ (Linse et al., 1991b), addition of K^+ (up to a concentration of 150 mM) to apo-CaM solution causes little chemical shift change to the backbone amide resonances throughout the protein (Linse et al., 1991a; data not shown), indicating that K^+ has a minimal effect on the structure of CaM. The reduced macroscopic Ca^{2+} binding constants in the presence of K^+ are, thus, a pure nonspecific ionic effect. Whereas, the binding of Mg^{2+} to apo-CaM induced much larger chemical

shift changes of the residues in the four Ca^{2+} -binding loops (Figure 1), indicating that Mg^{2+} binds to CaM with some specificity. It is likely that the decreased macroscopic Ca^{2+} binding constants observed in this study resulted from competition of Mg^{2+} for Ca^{2+} -binding sites as well as from a nonspecific ionic strength effect.

Mg²⁺ Ions Reduce the Affinity of CaM for Target Peptides. Much work has focused on the effect of bulk Mg^{2+} on the Ca^{2+} binding affinity of CaM (Haiech et al., 1981; Drabikowski et al., 1982; Iida & Potter, 1986; Milos et al., 1986). However, the functional role of Mg^{2+} ions on the target activation of CaM is largely unaddressed in the literature, mainly due to the general belief that Mg^{2+} does not play a significant role in this process. In addition, it is difficult to address the question of Mg^{2+} in the target activation of CaM since an excess amount of Mg^{2+} is used in the buffers under many *in vitro* assay conditions. Here, we used three synthesized peptides, encompassing the CaM-binding domains of MLCK, PDE, and CaD, to study the effect of Mg^{2+} on peptide-CaM complex formation. All three peptides contain one Trp residue, and the fluorescence of the Trp residue underwent significant changes in both its intensity and emission maximum upon binding to CaM as shown in Figure 3. Hence, the binding of these peptides to CaM at various concentrations of Mg^{2+} could be carefully examined.

Figure 3 shows fluorescence spectra of the target peptides in the presence and absence of CaM. For all three peptides, the maximum intensity appeared around 355 nm in the absence of CaM, corresponding to a fully solvent-exposed Trp residue. Fluorescence spectra of the free peptides were not affected by the addition of Mg^{2+} up to 100 mM (data not shown). Addition of CaM to each peptide solution led to a blue shift of the fluorescence spectra as well as to signal intensification, indicating that the Trp residue was in a more hydrophobic environment when the peptides were bound to CaM. Structure determinations of the CaM-MLCK peptide complexes have indeed shown that the Trp residue of the MLCK peptides is deeply buried in the hydrophobic interface formed between the hydrophobic cavity of CaM and hydrophobic residues from the peptide (Ikura et al., 1992; Meador et al., 1992). For high-affinity CaM targets such as

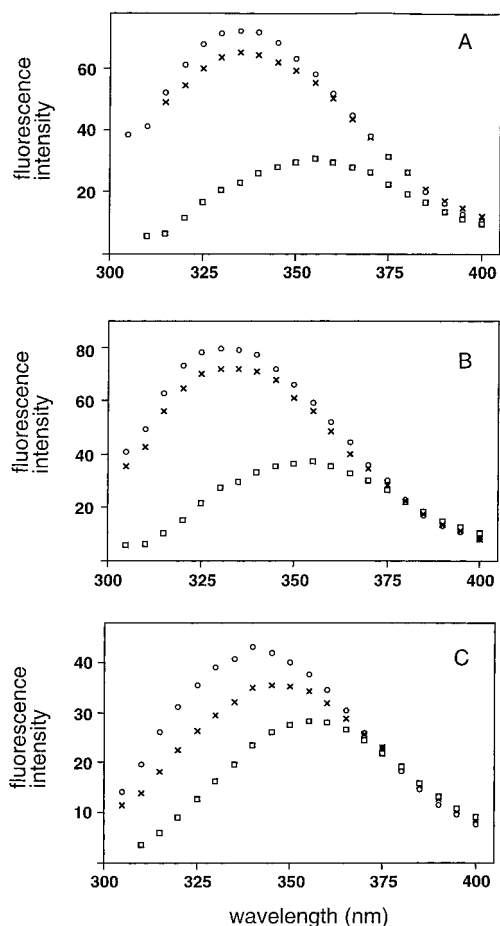


FIGURE 3: Fluorescence emission spectra of (A) the PDE peptide, (B) the MLCK peptide, and (C) the CaD peptide. In all three panels, the open squares (□) represent the emission spectra of the free peptides, the open circles (○) are spectra obtained with the peptides and calmodulin at a 1:1 ratio in the absence of Mg²⁺, and the crosses (×) denote spectra obtained with the peptides and calmodulin at a 1:1 ratio in the presence of 100 mM Mg²⁺.

the MLCK peptide and the PDE peptide, the addition of Mg²⁺ to the peptide–CaM complexes significantly reduced the signal intensity, although the wavelengths of the spectral maxima were unchanged when the concentration of Mg²⁺ increased (Figure 3A,B). With a weak CaM target such as the CaD peptide studied here, the addition of Mg²⁺ into the peptide–CaM complex not only reduced the signal intensity but also led to a red shift in the emission maximum (Figure 3C), suggesting that the Trp residue spends less time in the bound, hydrophobic environment of the complex.

Plots of relative fluorescence intensity, F/F_0 , as a function of the [CaM]:[peptide] ratio for all three peptides are shown in Figure 4. At various Mg²⁺ concentrations, the F/F_0 values increased with the addition of CaM and the changes continued till the [CaM]:[peptide] ratio reached ~1.0. The results indicated that all three peptides bound to Ca²⁺-saturated CaM with a 1:1 stoichiometry and furthermore that the binding stoichiometry was not affected by Mg²⁺. Earlier work with the same PDE peptide claimed that two PDE peptide molecules bound to one CaM molecule (Charbonneau et al., 1991), but the results from this study together with our earlier observation (Zhang et al., 1994) clearly indicate that the PDE peptide binds tightly to CaM with 1:1 stoichiometry. The saturated value of F/F_0 for all three peptides decreased when the concentration of Mg²⁺ increased (Figure 5), suggesting that Mg²⁺ ions reduce the affinities

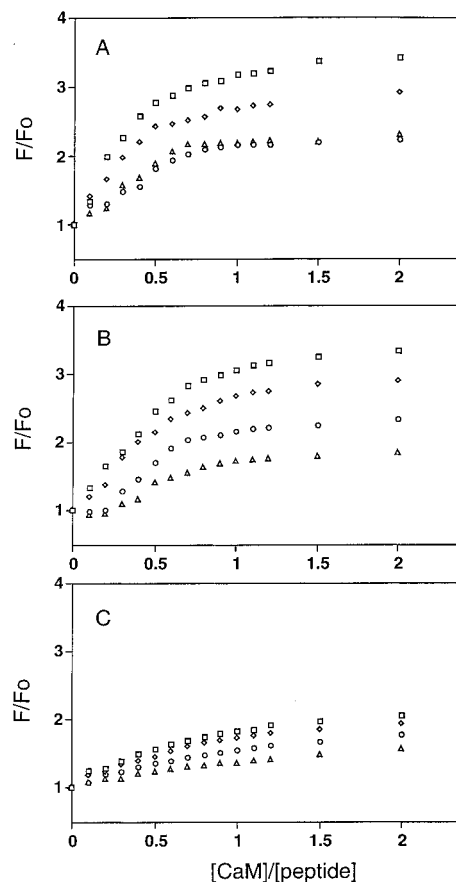
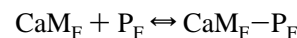


FIGURE 4: Plot of the relative fluorescence intensity (F/F_0) against the [CaM]:[peptide] ratio of (A) the PDE peptide, (B) the MLCK peptide, and (C) the CaD peptide. The symbols used are as follows: 0 (□), 10 (◇), 50 (○), and 100 (△) mM Mg²⁺.

of Ca₄–CaM for its target peptides. CD spectra of the Ca₄–CaM–peptide complexes with all three peptides were not distinguishable in the presence and absence of Mg²⁺ (data not shown), indicating that the second- and higher-order structures of the complexes were not disturbed by the addition of Mg²⁺.

Under the experimental conditions described here, virtually all CaM molecules are in the Ca²⁺-saturated form; hence, the binding reaction can be described as a simple equilibrium:



and known equations are

$$P_F = P_T / (1 + \text{CaM}_F k) \quad (1)$$

$$0 = P_F \text{CaM}_F k + \text{CaM}_F - iP_T \quad (2)$$

$$i = f \text{CaM}_T / P_T \quad (3)$$

where P_F , P_T , CaM_F , and CaM_T are concentrations of free peptide, total peptide, Ca²⁺-saturated CaM without peptide, and total CaM, respectively, and k is an association constant. For each set of variables, the Newton method was used to solve for the concentration of CaM_F at each titration point i (including a factor f for correcting the concentration ratio) from the equations (2). Then the fluorescence intensity at titration point i , F_i , can be calculated as

$$F_i = F_0 + (F_{\max} - F_0) P_F \text{CaM}_F k / P_T \quad (4)$$

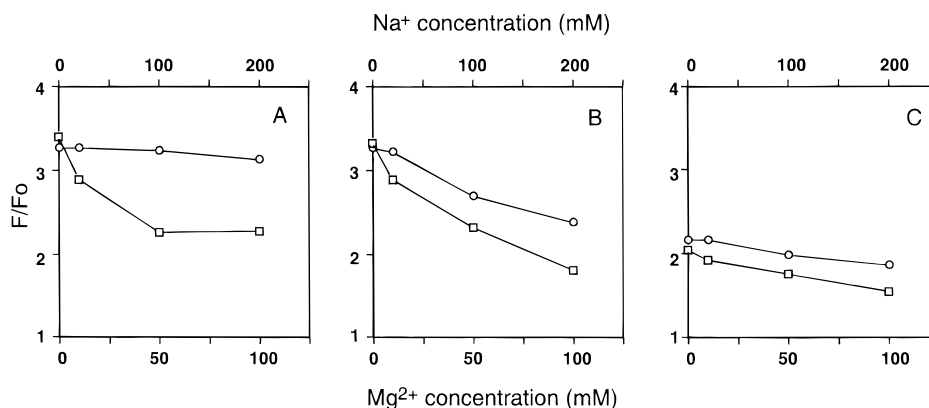


FIGURE 5: Plot of the relative fluorescence intensity (F/F_0) of peptides in a CaM-saturated state as a function of Mg^{2+} (\square) and Na^+ (\circ) concentrations (millimolar). Panels A–C represent data for the PDE peptide, the MLCK peptide, and the CaD peptide, respectively. The F/F_0 values for each peptide were taken at a $[CaM]:[peptide]$ ratio of 2:1. The maximum error of F/F_0 is ± 0.2 .

Table 2: Dissociation Constants [K_d (Nanomolar)]^a between CaM and Target Peptides in the Presence of Various Concentrations of Mg^{2+}

	K_d			
	0 mM Mg^{2+}	10 mM Mg^{2+}	50 mM Mg^{2+}	100 mM Mg^{2+}
MLCK peptide	2.0	7.0	80	78
PDE peptide	4.5	9.5	ND ^b	ND ^b
CaD peptide	890	950	1200	2000

^a Maximum error value of K_d is less than $\pm 15\%$. ^b ND means that the K_d is not estimated due to abnormal titration curves as described in the Results.

where F_0 is the observed fluorescence intensity of peptide without calmodulin and F_{max} is the saturated fluorescence intensity. Before using eq 4, the contribution to each F_i from the fluorescence intensity of calmodulin is subtracted. Each binding constant, k , is estimated from the best curve fitting of the experimental data points.

Table 2 shows dissociation constant ($K_d = 1/k$) values for each peptide in the presence of various concentrations of Mg^{2+} . Dissociation constants of the MLCK, PDE, and CaD peptides in the absence of Mg^{2+} were determined as 2.0×10^{-9} , 4.5×10^{-9} , and 8.9×10^{-7} M, respectively. These constants are in good agreement with the values reported earlier (Zhan et al., 1991; Blumenthal et al., 1985; Charbonneau et al., 1991). In the presence of Mg^{2+} , we observed significant increases in the dissociation constants of all three peptides for CaM. The titration curves of the PDE peptide at high Mg^{2+} concentrations displayed sigmoid-like shapes (Figure 3A), and we were not able to determine K_d values under such conditions by fitting the experimental data using eq 3 above. However, the decreased F/F_0 values at high Mg^{2+} concentrations shown in Figure 3A clearly indicate that the affinity of Ca^{2+} -saturated CaM for the PDE peptide was reduced significantly, compared to the value in the absence of Mg^{2+} . The lack of fluorescence intensity changes at low $[CaM]:[peptide]$ ratios suggests that Mg^{2+} inhibited the formation of the complex under these conditions.

To determine whether the Mg^{2+} effect observed in this study is a specific, intrinsic property of Mg^{2+} or rather is due to a nonspecific ionic effect on Ca^{2+} -saturated CaM, we repeated the fluorescence titration experiments with all three peptides but with Na^+ (at concentrations of 0, 20, 100, and 200 mM) instead of Mg^{2+} in the binding buffer. Figure 5 shows the F/F_0 values of all three peptides as a function

of Mg^{2+} or Na^+ concentrations. It is clear that the Na^+ effect on F/F_0 is different from that of Mg^{2+} . Unlike Mg^{2+} , Na^+ has essentially no effect on F/F_0 values for the MLCK peptide at ion concentrations up to 200 mM, indicating that the Mg^{2+} -induced decrease in the affinity of the MLCK peptide for CaM is specific (Figure 5A). For the PDE peptide, Mg^{2+} has a significantly larger effect on the peptide's affinity for CaM than does Na^+ , even though the F/F_0 values for the PDE peptide also decrease as a function of the Na^+ concentration (Figure 5B). Recently, another high-affinity CaM-binding domain, which is located about 90 amino acid residues carboxy-terminal to the one studied here, was identified for PDE (Sonnenburg et al., 1995). Earlier studies have shown that only one molecule of CaM binds to one monomer of PDE (Sharma & Wang, 1986). Hence, there is a possibility that *in vivo* both CaM-binding domains bind to one CaM molecule simultaneously as is the case found in the γ subunit of phosphorylase kinase (Dasgupta et al., 1989). Earlier NMR data have shown that the CaM–PDE peptide complex at a 1:1 ratio is not conformationally homogeneous (Zhang et al., 1994). In addition, the binding of the PDE peptide described here to CaM is not strictly Ca^{2+} -dependent at low ionic strengths (Charbonnen et al., 1991). These unusual binding properties of the PDE peptide may account for the partial ionic strength-dependent affinity decrease depicted in Figure 5B. For weaker CaM targets like the CaD peptide, the effect of Mg^{2+} and Na^+ on CaM's target binding is more similar, although Mg^{2+} shows a somewhat larger effect (Figure 5C), indicating that pure ionic effects are probably playing more important roles in this case. It has been noticed before that the formation of complexes between CaM and its weaker targets could be salt concentration-dependent (Chapman et al., 1991). The results shown in Figure 5 indicate that, at least for some of the targets, the Mg^{2+} -induced affinity reduction on target binding by CaM is an intrinsic property of the protein, rather than a nonspecific ionic effect.

DISCUSSION

In eukaryotic cells, the concentration of CaM is in the range of $3\text{--}20 \times 10^{-6}$ M. A rise in the intracellular Ca^{2+} concentration converts CaM into a Ca^{2+} -saturated form. However, when cells are in a resting state, Ca^{2+} -free CaM is at least partially saturated with Mg^{2+} due to the existence of a millimolar concentration of Mg^{2+} . It is, therefore, important to characterize the Mg^{2+} binding properties of apo-

CaM and to understand the effect of Mg²⁺ on the conformation of the protein. Such knowledge will also shed light on the mechanism of the specific response of CaM toward narrow concentration fluctuations of Ca²⁺ in the presence of high levels of Mg²⁺.

The NMR results given here indicate the following. (i) Mg²⁺ shares the same binding sites with Ca²⁺ since only the resonances from the Ca²⁺-binding sites experience line width and chemical shift changes. Similar behavior is observed for the N-terminal half-domain of yeast calmodulin when it is titrated with Mg²⁺ (S. Ohki and K. Hikichi, unpublished data). However, in all likelihood, Mg²⁺ does not use the same ligands as Ca²⁺ as the last three residues in each 12-residue Ca²⁺-binding loop do not undergo chemical shift changes during titration. The side chains of the negatively charged Asp residues (e.g. D20, D22, and D24 in site I) in each Ca²⁺-binding loop are the ligands most likely to interact with Mg²⁺. This is not unusual as Mg²⁺ rarely uses neutral oxygen donors such as carbonyls and hydroxyls as ligands, and Mg²⁺ always forms regular octahedral coordination spheres with a coordination number of six (da Silva & Williams, 1991). Ca²⁺, however, interacts with both negatively charged carboxyls and neutral carbonyls and hydroxyls, and the seven oxygen ligands in each Ca²⁺-binding site are arranged in a distorted pentagonal bipyramidal geometry around the Ca²⁺ ion (Babu et al., 1988).

(ii) Mg²⁺ binds to Ca²⁺-binding sites I and IV first, and then to site II. Ca²⁺-binding site III displays the lowest affinity for Mg²⁺. Mg²⁺ titration of scallop CaM using one-dimensional ¹H-NMR spectroscopy supports the present NMR results (Ohki et al., 1991; S. Ohki, U. Iwamoto, and K. Hikichi, unpublished data). During Mg²⁺ titration of scallop apo-CaM, the downfield-shifted α¹H resonance of T26 in site I and the aromatic protons of Y138 in site IV change at lower Mg²⁺ concentrations and the changes continue until the [Mg²⁺]:[CaM] ratio reaches ~5, consistent with the result presented here (sites I and IV bind Mg²⁺ first). At intermediate Mg²⁺ concentrations ([Mg²⁺]:[CaM] ratios from ~1.5 to ~8), the downfield-shifted α¹H resonance of D64 in site II changes to a slightly lower field position. Also, the downfield-shifted α¹H resonance of F99 (Y in *X. laevis* CaM) in site III changes at higher Mg²⁺ concentrations (10 ≤ [Mg²⁺]:[CaM] ≤ 40), further indicating that site III has the weakest affinity for Mg²⁺.

As site III and IV are the high-affinity sites for Ca²⁺, this result is rather unexpected, especially given the fact that a pair of high-affinity Ca²⁺-binding sites (site III and IV) of troponin C are also the high-affinity sites for Mg²⁺ (Potter & Gergely, 1975). However, Mg²⁺ and Ca²⁺ may not necessarily share the same binding preference since the coordination chemistry of these two ions is fundamentally different, as discussed above. Ca²⁺ binding to CaM is a cooperative event, and such cooperativity partly arises from close helix pairing between the two EF hands in each domain (Zhang et al., 1995). The binding of Ca²⁺ to the protein leads to global conformational changes in each domain, and such conformational changes link the two Ca²⁺-binding sites in each domain together. The Mg²⁺ binding results in only localized conformational changes in the Ca²⁺-binding sites of the protein (see below). Therefore, the affinity of each Ca²⁺-binding site for Mg²⁺ in CaM is dependent only on its local sequence. In troponin C, however, both Mg²⁺ and Ca²⁺ binding to the Mg²⁺ and Ca²⁺ sites in the C-terminal domain

induce tertiary structural changes of the protein, and the metal ions serve as structural stabilizers of the protein instead of as a functional regulator as in the case in CaM (Zot & Potter, 1987). The detection of a pair of high-affinity Mg²⁺-binding sites in CaM using high-resolution NMR spectroscopy presented in this work agrees with earlier results obtained from a mass spectrometric study (Lafitte et al., 1995), although the mass spectrometric study failed to show that Mg²⁺ and Ca²⁺ share the binding sites. The existence of a high-affinity (K_d on the order of 10⁻³ M) Mg²⁺-binding site in the N-terminal domain of CaM has also been demonstrated earlier using ²⁵Mg NMR spectroscopy (Tsai, 1987). The failure to detect the high-affinity Mg²⁺ site in the C-terminal domain of CaM could be due to the large line width of the bound Mg²⁺ signal, making it indistinguishable from the baseline. Earlier thermodynamic studies of CaM have shown that Mg²⁺ binding to apo-CaM did not affect the stability of the third EF-hand module of the protein (Tsalkova & Privalov, 1985), in line with our result showing that Mg²⁺ shows very little binding to site III.

(iii) Mg²⁺-induced conformational changes are localized only in the metal-binding sites, and the rest of the protein remains unchanged. Hence, CaM specifically responds to cellular Ca²⁺ concentration fluctuations only, and a high intracellular Mg²⁺ concentration does not affect the function of the protein. Moreover, Mg²⁺-free, Ca²⁺-free and Mg²⁺-bound, Ca²⁺-free CaM should have same overall conformation except in the areas of the flexible metal ion-binding sites. This notion reinforces the physiological relevance of the Ca²⁺-free CaM structure determined earlier in the absence of Mg²⁺ (Kuboniwa et al., 1995; Zhang et al., 1995).

Figure 2B shows the ¹H-¹⁵N HSQC spectrum of Ca²⁺-saturated CaM in the presence of Mg²⁺, and this spectrum is essentially identical to that of the Mg²⁺-free, Ca²⁺-saturated protein (Ikura et al., 1990; data not shown), indicating that Ca²⁺-saturated CaM has the same three-dimensional conformation both in the absence and in the presence of excess Mg²⁺ (up to 10 mM). This indicates that, once CaM is activated upon the rising of the intracellular Ca²⁺ concentration, Mg²⁺ does not affect the structure of the Ca²⁺-saturated CaM. The results of Figure 2B suggest that the interaction between Mg²⁺ and Ca₄-CaM is relatively weak and does not disturb the conformation of the protein in an appreciable manner.

The Mg²⁺-induced affinity decreases of the target peptides for CaM, seen here, indicate that the binding between Ca²⁺-saturated CaM and targets in living cells may be less avid than has been reported from *in vitro* studies in the absence of Mg²⁺. It is well-known that the intracellular Ca²⁺ concentration increases from 10⁻⁷ M at the resting state to 10⁻⁶ M or higher upon stimulation. Appearance/disappearance of target activity is modulated by the fluctuations of the intracellular Ca²⁺ concentration. If we assume a K_d value of 10⁻¹⁰–10⁻⁹ M between a target and CaM, and that concentrations of the target and CaM in the cells are 1 and 10 μM, respectively, a theoretical simulation indicates that nearly 100% of the target would form a complex with CaM even when intracellular Ca²⁺ concentration is 10⁻⁷ M, a result in contradiction with experimental observations. Therefore, bulk cellular Mg²⁺ may partially allow CaM to respond to narrow physiological cellular Ca²⁺ concentration changes by decreasing the affinities between CaM and some of its targets. This Mg²⁺ effect is probably related to a recently

proposed mechanism for the calcium regulation of CaM-target activation, involving an intermediate species Ca₂-CaM-target (two Ca²⁺ ions binding to the C-terminal half-domain of CaM) at an intermediate Ca²⁺ concentration (Ohki et al., 1993; Bayley et al., 1996). Certainly, there could be many other factors that contribute to the specific response of CaM to cellular Ca²⁺ concentration fluctuations. The limited accessibility of the CaM-binding domain in the intact target proteins would lead to a lower affinity between the intact enzyme and CaM compared to the affinity measured using a synthetic peptide corresponding to the CaM-binding domain (Goldberg et al., 1996). Competition between many target enzymes for CaM in living cells would also decrease the effective CaM concentration for each individual target enzyme. Uneven distribution of Ca²⁺ and CaM in a living cell would provide another controlling mechanism of various CaM-dependent enzymes.

In summary, our results have shown that CaM has one high-affinity Mg²⁺-binding site in each domain of the protein, and the Mg²⁺-binding sites are located within the Ca²⁺-binding sites of CaM. The binding of Mg²⁺ does not change the structures of CaM at a significant level both in the absence and in the presence of Ca²⁺. Some of the CaM targets display decreased affinities for CaM in the presence of an excess amount of Mg²⁺.

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