

# Identification of Mg<sup>2+</sup>-Binding Sites and the Role of Mg<sup>2+</sup> on Target Recognition by Calmodulin<sup>†</sup>

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Received November 5, 1996; Revised Manuscript Received January 10, 1997<sup>⊗</sup>

**ABSTRACT:** The binding of Mg<sup>2+</sup> to calmodulin (CaM) and the effect of Mg<sup>2+</sup> on the binding of Ca<sup>2+</sup>–CaM to target peptides were examined using two-dimensional nuclear magnetic resonance and fluorescence spectroscopic techniques. We found that Mg<sup>2+</sup> preferentially binds to Ca<sup>2+</sup>-binding sites I and IV of CaM in the absence of Ca<sup>2+</sup> and that Ca<sup>2+</sup>-binding site III displays the lowest affinity for Mg<sup>2+</sup>. In contrast to the marked structural transitions induced by Ca<sup>2+</sup> binding, Mg<sup>2+</sup> binding causes only localized conformational changes within the four Ca<sup>2+</sup>-binding loops of CaM. Therefore, Mg<sup>2+</sup> does not seem to be able to cause significant structural effects required for the interaction of CaM with target proteins. The presence of excess Mg<sup>2+</sup> (up to 10 mM) does not change the order and cooperativity of Ca<sup>2+</sup> binding to CaM, and as expected, the structure of Ca<sup>2+</sup>-saturated CaM is not affected by the presence of Mg<sup>2+</sup>. However, we found that the binding of Ca<sup>2+</sup>-saturated CaM to target peptides is affected by Mg<sup>2+</sup> with the binding affinity decreasing as the Mg<sup>2+</sup> 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 Mg<sup>2+</sup> is approximately 40-fold lower than that seen in the absence of Mg<sup>2+</sup>, and a similar response was observed for the PDE peptide. The affinity of the CaD peptide for CaM also shows a Mg<sup>2+</sup> dependence, though to a much lower magnitude. The Mg<sup>2+</sup>-dependent decrease in the affinities between CaM and its target peptides is an intrinsic property of Mg<sup>2+</sup> rather than a nonspecific ionic effect, as other metal ions such as Na<sup>+</sup> do not completely replicate the effect of Mg<sup>2+</sup>. The inhibitory effect of Mg<sup>2+</sup> on the formation of complexes between CaM and its targets may contribute to the specificity of CaM in target activation in response to cellular Ca<sup>2+</sup> concentration fluctuations.

The ubiquitous eukaryotic protein calmodulin (CaM)<sup>1</sup> is responsible for converting the intracellular Ca<sup>2+</sup> signal into a wide range of physiological events. Successful signal conversion is achieved by transforming Ca<sup>2+</sup>-free CaM into its Ca<sup>2+</sup>-saturated form as the intracellular Ca<sup>2+</sup> concentration rises. The Ca<sup>2+</sup>-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).

Ca<sup>2+</sup>-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 Ca<sup>2+</sup> binding. The 6 N-terminal residues in each of the four 12-residue Ca<sup>2+</sup>-binding sites (sites I–IV) are highly mobile (Zhang et al., 1995; Tjandra et al., 1995; M. Zhang, unpublished). Ca<sup>2+</sup> 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 Ca<sup>2+</sup> sites was observed (Andersson et al., 1983; Ikura et al., 1983; Dalgarno et al., 1984). The binding of Ca<sup>2+</sup> 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 Ca<sup>2+</sup>-free state (Zhang et al., 1995; Kuboniwa et al., 1995) to almost perpendicular in the Ca<sup>2+</sup>-saturated state (Babu et al., 1988). Thus, both domains of CaM undergo a change from a four-helix bundle-like “closed” conformation in the Ca<sup>2+</sup>-free state to a well-separated “open” conformation in the Ca<sup>2+</sup>-saturated state (Zhang et al., 1995; Kuboniwa et al., 1995; Babu et al., 1988). The Ca<sup>2+</sup>-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 Ca<sup>2+</sup>-free CaM, a large solvent-exposed hydrophobic surface with a deep cavity is observed in each domain of Ca<sup>2+</sup>-saturated CaM (Zhang et al., 1995; Babu et al., 1988). The formation of the hydrophobic cavity is the most important structural feature

<sup>†</sup> Part of this work is sponsored by an RGC grant to M.Z. from the Research Grant Committee of Hong Kong. M.I. is a Scholar of the Medical Research Council of Canada.

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<sup>⊗</sup> Abstract published in *Advance ACS Abstracts*, March 15, 1997.

<sup>1</sup> Abbreviations: Br<sub>2</sub>BAPTA, 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  $\text{Ca}^{2+}$  (Ikura et al., 1992; Meador et al., 1992, 1993).

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

## MATERIALS AND METHODS

**Proteins and Peptides.** Uniformly  $^{15}\text{N}$ -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  $^{15}\text{N}$ -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  $^{15}\text{N}$ -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  $\text{Mg}^{2+}$  until 10 equiv of  $\text{Mg}^{2+}$  was added to the sample and then subjected to  $\text{Ca}^{2+}$  titration. The other  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free sample was prepared for  $\text{Ca}^{2+}$  titration in order to allow a measure of the effect of  $\text{Mg}^{2+}$  on  $\text{Ca}^{2+}$  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).

**$\text{Ca}^{2+}$  Binding.** Each calmodulin fragment (25  $\mu\text{M}$ ) was titrated with  $\text{Ca}^{2+}$  in 25 mM Tris buffer containing various concentrations of  $\text{Mg}^{2+}$  (0, 10, 50, and 100 mM), 25  $\mu\text{M}$   $\text{Br}_2\text{BAPTA}$ , 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.  $\text{Ca}^{2+}$  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  $\text{Mg}^{2+}$  (0, 10, 50, and 100 mM) were used to study the effect of  $\text{Mg}^{2+}$  on the formation of the complexes. In another set of CaM-peptide binding experiments, the complexes containing various concentrations of  $\text{Na}^+$  (0, 20, 100, and 200 mM) instead of  $\text{Mg}^{2+}$  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  $\mu\text{M}$  (for the MLCK peptide and the PDE peptide) and 8.0  $\mu\text{M}$  (for the CaD peptide). The binding reaction was carried out in a buffer containing 25 mM Tris, 100 mM KCl, and 1 mM  $\text{CaCl}_2$  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

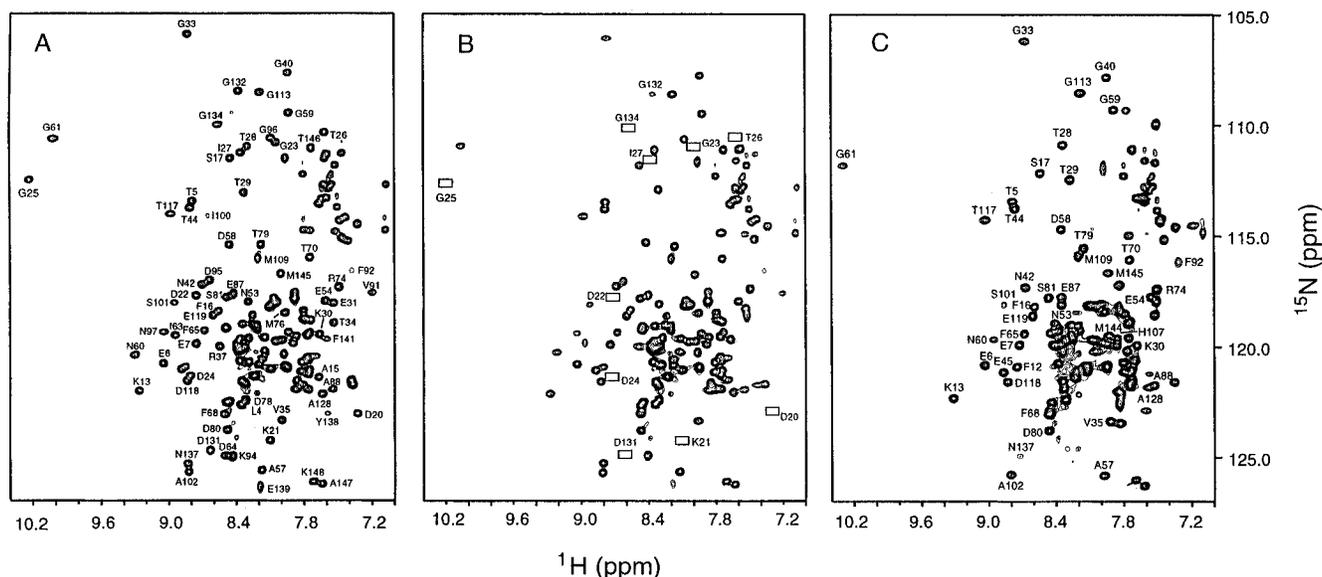


FIGURE 1: <sup>1</sup>H–<sup>15</sup>N HSQC spectra of uniformly <sup>15</sup>N-labeled CaM with different concentrations of Mg<sup>2+</sup> and Ca<sup>2+</sup> ions. (A) Mg<sup>2+</sup>-free, Ca<sup>2+</sup>-free CaM; most of the resonances are labeled with their residue name and number. (B) CaM with no Ca<sup>2+</sup> and 1 equiv of Mg<sup>2+</sup>; the open boxes highlight the resonances that disappear upon the addition of 1 equiv of Mg<sup>2+</sup> to the sample. (C) Ca<sup>2+</sup>-free protein with 10 equiv of Mg<sup>2+</sup>; 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

*Mg<sup>2+</sup> Ions Bind Preferentially to Ca<sup>2+</sup>-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 Ca<sup>2+</sup> and Mg<sup>2+</sup> sites in the C-terminal domain of the protein (Potter & Gergely, 1975). Several points are still unclear: (a) the existence of specific Mg<sup>2+</sup> site(s) in CaM, (b) the stoichiometry of Mg<sup>2+</sup> binding to CaM, (c) the site preference of Mg<sup>2+</sup> in CaM, and (d) the conformational effects on CaM of Mg<sup>2+</sup> binding. In an attempt to resolve the above issues, we performed Mg<sup>2+</sup> titration experiments on apo-CaM by recording <sup>1</sup>H–<sup>15</sup>N HSQC spectra of uniformly <sup>15</sup>N-labeled CaM for which complete backbone assignments are available both in the Ca<sup>2+</sup>-free state (Zhang et al., 1995) and in the Ca<sup>2+</sup>-saturated state (Ikura et al., 1990).

Figure 1A shows the backbone <sup>1</sup>H–<sup>15</sup>N HSQC spectrum of Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free CaM at pH 6.5. When the first equivalent of Mg<sup>2+</sup> was added to the protein, the resonances corresponding to the first and fourth Ca<sup>2+</sup>-binding sites (highlighted with open boxes in Figure 1B) underwent substantial line broadening, whereas the resonances from the second and third Ca<sup>2+</sup>-binding sites remained almost unchanged. Such line broadening arises from the fact that the Mg<sup>2+</sup>-bound and the Mg<sup>2+</sup>-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 Mg<sup>2+</sup> 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 Mg<sup>2+</sup> reached 10 equiv, a new set of resonances were observed (Figure 1C); these resonances presumably belong to the residues from the Mg<sup>2+</sup>-bound Ca<sup>2+</sup> 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 Mg<sup>2+</sup> binding induced conformational changes localized only to the Ca<sup>2+</sup>-binding loops of the protein.

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

Using a competitive Ca<sup>2+</sup>-binding dye, Br<sub>2</sub>BAPTA, we measured the Ca<sup>2+</sup> binding constants of calmodulin in the presence of various concentrations of Mg<sup>2+</sup>. In order to

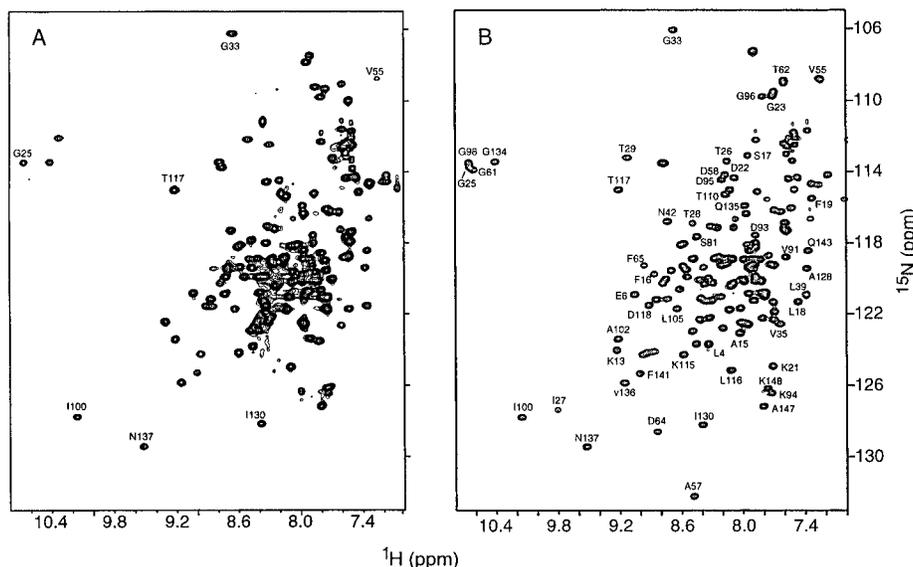


FIGURE 2:  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of CaM in the presence of 1.6 (A) and 4.5 equiv (B) of  $\text{Ca}^{2+}$ . The protein sample contains 10 mM  $\text{Mg}^{2+}$ . For clarity, only selected residues are labeled [for further details, see Ikura et al. (1990)].

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

[ $\text{Mg}^{2+}$ ] (mM)	TR1C <sup>a</sup>		TR2C <sup>a</sup>	
	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

<sup>a</sup> The uncertainties are  $\pm 0.2$  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  $\text{Ca}^{2+}$  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  $\text{Mg}^{2+}$  up to 10 mM did not have a significant effect on the  $\text{Ca}^{2+}$  binding affinity and cooperativity of CaM, which is in good agreement with the results of the NMR studies described above. However, at higher  $\text{Mg}^{2+}$  concentrations, a noticeable decrease in  $\text{Ca}^{2+}$  affinity was observed for the C-terminal domain of the protein, while the N-terminal domain of the protein displayed a lower sensitivity to  $\text{Mg}^{2+}$  concentration changes. At a  $\text{Mg}^{2+}$  concentration of 100 mM, both the N-terminal and the C-terminal domains had similar  $\text{Ca}^{2+}$  binding constants.

In order to answer the question of whether the  $\text{Mg}^{2+}$ -induced decrease of the macroscopic  $\text{Ca}^{2+}$  binding constant is a result of (a) direct competition between  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  or (b) a pure nonspecific ionic effect, we have compared the chemical shift changes of the backbone amides induced by the addition of  $\text{K}^+$  and  $\text{Mg}^{2+}$ . Similar to what was found in a related  $\text{Ca}^{2+}$ -binding protein calbindin  $\text{D}_{9\text{K}}$  (Linse et al., 1991b), addition of  $\text{K}^+$  (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  $\text{K}^+$  has a minimal effect on the structure of CaM. The reduced macroscopic  $\text{Ca}^{2+}$  binding constants in the presence of  $\text{K}^+$  are, thus, a pure nonspecific ionic effect. Whereas, the binding of  $\text{Mg}^{2+}$  to apo-CaM induced much larger chemical

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

*Mg<sup>2+</sup> Ions Reduce the Affinity of CaM for Target Peptides.* Much work has focused on the effect of bulk  $\text{Mg}^{2+}$  on the  $\text{Ca}^{2+}$  binding affinity of CaM (Haiech et al., 1981; Drabikowski et al., 1982; Iida & Potter, 1986; Milos et al., 1986). However, the functional role of  $\text{Mg}^{2+}$  ions on the target activation of CaM is largely unaddressed in the literature, mainly due to the general belief that  $\text{Mg}^{2+}$  does not play a significant role in this process. In addition, it is difficult to address the question of  $\text{Mg}^{2+}$  in the target activation of CaM since an excess amount of  $\text{Mg}^{2+}$  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  $\text{Mg}^{2+}$  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  $\text{Mg}^{2+}$  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  $\text{Mg}^{2+}$  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

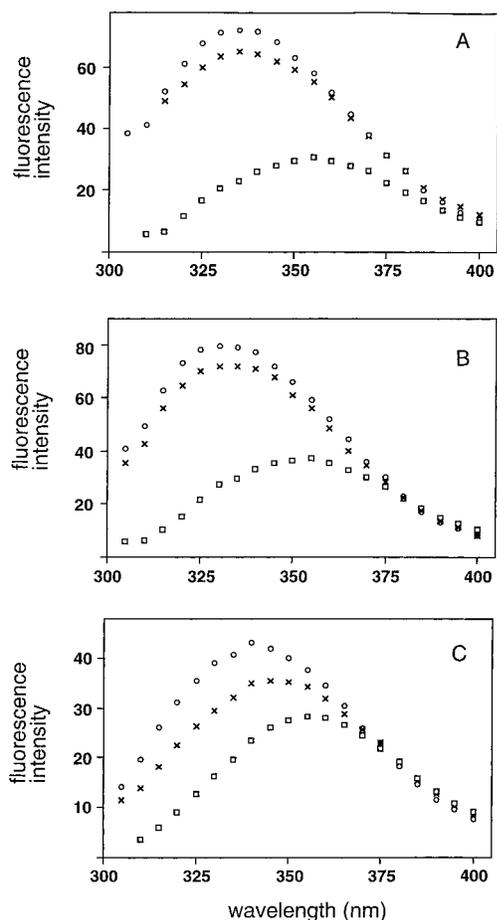


FIGURE 3: Fluorescence emission spectra of (A) the PDE peptide, (B) the MLCK peptide, and (C) the CaD peptide. In all three panels, the open squares (□) represent the emission spectra of the free peptides, the open circles (○) are spectra obtained with the peptides and calmodulin at a 1:1 ratio in the absence of Mg<sup>2+</sup>, and the crosses (×) denote spectra obtained with the peptides and calmodulin at a 1:1 ratio in the presence of 100 mM Mg<sup>2+</sup>.

the MLCK peptide and the PDE peptide, the addition of Mg<sup>2+</sup> to the peptide–CaM complexes significantly reduced the signal intensity, although the wavelengths of the spectral maxima were unchanged when the concentration of Mg<sup>2+</sup> increased (Figure 3A,B). With a weak CaM target such as the CaD peptide studied here, the addition of Mg<sup>2+</sup> 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 Mg<sup>2+</sup> 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 Ca<sup>2+</sup>-saturated CaM with a 1:1 stoichiometry and furthermore that the binding stoichiometry was not affected by Mg<sup>2+</sup>. 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 Mg<sup>2+</sup> increased (Figure 5), suggesting that Mg<sup>2+</sup> ions reduce the affinities

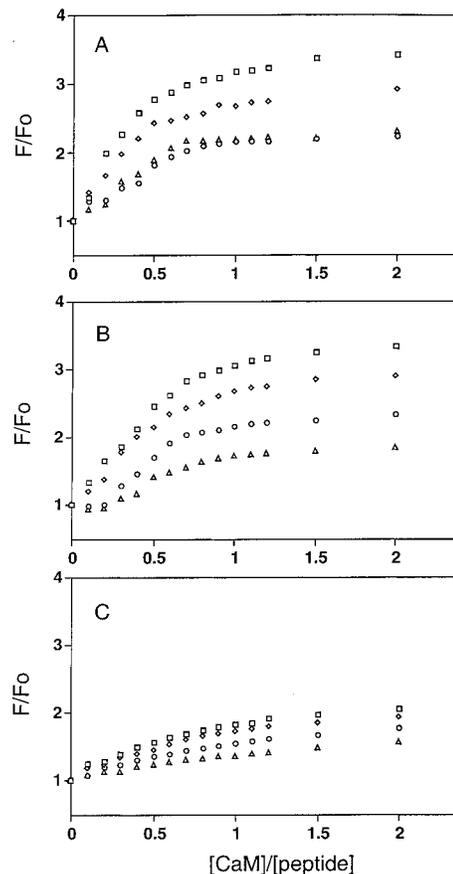
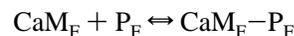


FIGURE 4: Plot of the relative fluorescence intensity ( $F/F_0$ ) against the [CaM]:[peptide] ratio of (A) the PDE peptide, (B) the MLCK peptide, and (C) the CaD peptide. The symbols used are as follows: 0 (□), 10 (◇), 50 (○), and 100 (△) mM Mg<sup>2+</sup>.

of Ca<sub>4</sub>–CaM for its target peptides. CD spectra of the Ca<sub>4</sub>–CaM–peptide complexes with all three peptides were not distinguishable in the presence and absence of Mg<sup>2+</sup> (data not shown), indicating that the second- and higher-order structures of the complexes were not disturbed by the addition of Mg<sup>2+</sup>.

Under the experimental conditions described here, virtually all CaM molecules are in the Ca<sup>2+</sup>-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  $P_F$ ,  $P_T$ ,  $\text{CaM}_F$ , and  $\text{CaM}_T$  are concentrations of free peptide, total peptide, Ca<sup>2+</sup>-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  $\text{CaM}_F$  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

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

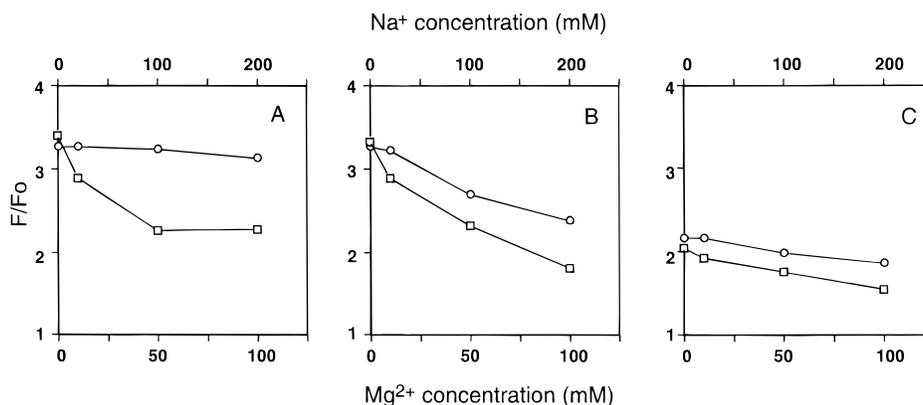


FIGURE 5: Plot of the relative fluorescence intensity ( $F/F_0$ ) of peptides in a CaM-saturated state as a function of  $Mg^{2+}$  ( $\square$ ) and  $Na^+$  ( $\circ$ ) concentrations (millimolar). Panels A–C represent data for the PDE peptide, the MLCK peptide, and the CaD peptide, respectively. The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

Table 2: Dissociation Constants [ $K_d$  (Nanomolar)]<sup>a</sup> 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 <sup>b</sup>	ND <sup>b</sup>
CaD peptide	890	950	1200	2000

<sup>a</sup> Maximum error value of  $K_d$  is less than  $\pm 15\%$ . <sup>b</sup> 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  $2.0 \times 10^{-9}$ ,  $4.5 \times 10^{-9}$ , and  $8.9 \times 10^{-7}$  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  $\gamma$  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 Mg<sup>2+</sup> 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 Ca<sup>2+</sup> in the presence of high levels of Mg<sup>2+</sup>.

The NMR results given here indicate the following. (i) Mg<sup>2+</sup> shares the same binding sites with Ca<sup>2+</sup> since only the resonances from the Ca<sup>2+</sup>-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 Mg<sup>2+</sup> (S. Ohki and K. Hikichi, unpublished data). However, in all likelihood, Mg<sup>2+</sup> does not use the same ligands as Ca<sup>2+</sup> as the last three residues in each 12-residue Ca<sup>2+</sup>-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 Ca<sup>2+</sup>-binding loop are the ligands most likely to interact with Mg<sup>2+</sup>. This is not unusual as Mg<sup>2+</sup> rarely uses neutral oxygen donors such as carbonyls and hydroxyls as ligands, and Mg<sup>2+</sup> always forms regular octahedral coordination spheres with a coordination number of six (da Silva & Williams, 1991). Ca<sup>2+</sup>, however, interacts with both negatively charged carboxyls and neutral carbonyls and hydroxyls, and the seven oxygen ligands in each Ca<sup>2+</sup>-binding site are arranged in a distorted pentagonal bipyramidal geometry around the Ca<sup>2+</sup> ion (Babu et al., 1988).

(ii) Mg<sup>2+</sup> binds to Ca<sup>2+</sup>-binding sites I and IV first, and then to site II. Ca<sup>2+</sup>-binding site III displays the lowest affinity for Mg<sup>2+</sup>. Mg<sup>2+</sup> titration of scallop CaM using one-dimensional <sup>1</sup>H-NMR spectroscopy supports the present NMR results (Ohki et al., 1991; S. Ohki, U. Iwamoto, and K. Hikichi, unpublished data). During Mg<sup>2+</sup> titration of scallop apo-CaM, the downfield-shifted α<sup>1</sup>H resonance of T26 in site I and the aromatic protons of Y138 in site IV change at lower Mg<sup>2+</sup> concentrations and the changes continue until the [Mg<sup>2+</sup>]:[CaM] ratio reaches ~5, consistent with the result presented here (sites I and IV bind Mg<sup>2+</sup> first). At intermediate Mg<sup>2+</sup> concentrations ([Mg<sup>2+</sup>]:[CaM] ratios from ~1.5 to ~8), the downfield-shifted α<sup>1</sup>H resonance of D64 in site II changes to a slightly lower field position. Also, the downfield-shifted α<sup>1</sup>H resonance of F99 (Y in *X. laevis* CaM) in site III changes at higher Mg<sup>2+</sup> concentrations (10 ≤ [Mg<sup>2+</sup>]:[CaM] ≤ 40), further indicating that site III has the weakest affinity for Mg<sup>2+</sup>.

As site III and IV are the high-affinity sites for Ca<sup>2+</sup>, this result is rather unexpected, especially given the fact that a pair of high-affinity Ca<sup>2+</sup>-binding sites (site III and IV) of troponin C are also the high-affinity sites for Mg<sup>2+</sup> (Potter & Gergely, 1975). However, Mg<sup>2+</sup> and Ca<sup>2+</sup> may not necessarily share the same binding preference since the coordination chemistry of these two ions is fundamentally different, as discussed above. Ca<sup>2+</sup> 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 Ca<sup>2+</sup> to the protein leads to global conformational changes in each domain, and such conformational changes link the two Ca<sup>2+</sup>-binding sites in each domain together. The Mg<sup>2+</sup> binding results in only localized conformational changes in the Ca<sup>2+</sup>-binding sites of the protein (see below). Therefore, the affinity of each Ca<sup>2+</sup>-binding site for Mg<sup>2+</sup> in CaM is dependent only on its local sequence. In troponin C, however, both Mg<sup>2+</sup> and Ca<sup>2+</sup> binding to the Mg<sup>2+</sup> and Ca<sup>2+</sup> 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 Mg<sup>2+</sup>-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 Mg<sup>2+</sup> and Ca<sup>2+</sup> share the binding sites. The existence of a high-affinity ( $K_d$  on the order of 10<sup>-3</sup> M) Mg<sup>2+</sup>-binding site in the N-terminal domain of CaM has also been demonstrated earlier using <sup>25</sup>Mg NMR spectroscopy (Tsai, 1987). The failure to detect the high-affinity Mg<sup>2+</sup> site in the C-terminal domain of CaM could be due to the large line width of the bound Mg<sup>2+</sup> signal, making it indistinguishable from the baseline. Earlier thermodynamic studies of CaM have shown that Mg<sup>2+</sup> 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 Mg<sup>2+</sup> shows very little binding to site III.

(iii) Mg<sup>2+</sup>-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 Ca<sup>2+</sup> concentration fluctuations only, and a high intracellular Mg<sup>2+</sup> concentration does not affect the function of the protein. Moreover, Mg<sup>2+</sup>-free, Ca<sup>2+</sup>-free and Mg<sup>2+</sup>-bound, Ca<sup>2+</sup>-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 Ca<sup>2+</sup>-free CaM structure determined earlier in the absence of Mg<sup>2+</sup> (Kuboniwa et al., 1995; Zhang et al., 1995).

Figure 2B shows the <sup>1</sup>H-<sup>15</sup>N HSQC spectrum of Ca<sup>2+</sup>-saturated CaM in the presence of Mg<sup>2+</sup>, and this spectrum is essentially identical to that of the Mg<sup>2+</sup>-free, Ca<sup>2+</sup>-saturated protein (Ikura et al., 1990; data not shown), indicating that Ca<sup>2+</sup>-saturated CaM has the same three-dimensional conformation both in the absence and in the presence of excess Mg<sup>2+</sup> (up to 10 mM). This indicates that, once CaM is activated upon the rising of the intracellular Ca<sup>2+</sup> concentration, Mg<sup>2+</sup> does not affect the structure of the Ca<sup>2+</sup>-saturated CaM. The results of Figure 2B suggest that the interaction between Mg<sup>2+</sup> and Ca<sub>4</sub>-CaM is relatively weak and does not disturb the conformation of the protein in an appreciable manner.

The Mg<sup>2+</sup>-induced affinity decreases of the target peptides for CaM, seen here, indicate that the binding between Ca<sup>2+</sup>-saturated CaM and targets in living cells may be less avid than has been reported from *in vitro* studies in the absence of Mg<sup>2+</sup>. It is well-known that the intracellular Ca<sup>2+</sup> concentration increases from 10<sup>-7</sup> M at the resting state to 10<sup>-6</sup> M or higher upon stimulation. Appearance/disappearance of target activity is modulated by the fluctuations of the intracellular Ca<sup>2+</sup> concentration. If we assume a  $K_d$  value of 10<sup>-10</sup>–10<sup>-9</sup> 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 Ca<sup>2+</sup> concentration is 10<sup>-7</sup> M, a result in contradiction with experimental observations. Therefore, bulk cellular Mg<sup>2+</sup> may partially allow CaM to respond to narrow physiological cellular Ca<sup>2+</sup> concentration changes by decreasing the affinities between CaM and some of its targets. This Mg<sup>2+</sup> effect is probably related to a recently

proposed mechanism for the calcium regulation of CaM–target activation, involving an intermediate species Ca<sub>2</sub>–CaM–target (two Ca<sup>2+</sup> ions binding to the C-terminal half-domain of CaM) at an intermediate Ca<sup>2+</sup> 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 Ca<sup>2+</sup> 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 Ca<sup>2+</sup> 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 Mg<sup>2+</sup>-binding site in each domain of the protein, and the Mg<sup>2+</sup>-binding sites are located within the Ca<sup>2+</sup>-binding sites of CaM. The binding of Mg<sup>2+</sup> does not change the structures of CaM at a significant level both in the absence and in the presence of Ca<sup>2+</sup>. Some of the CaM targets display decreased affinities for CaM in the presence of an excess amount of Mg<sup>2+</sup>.

#### ACKNOWLEDGMENT

We thank Professor Hans J. Vogel at the University of Calgary for providing the CaD and MLCK peptides used in this work, Professor Michio Yazawa at Hokkaido University for providing scallop calmodulin fragments, Professor Kunio Hikichi at Hokkaido University for sharing his unpublished results, and Dr. Frank Delaglio at the NIH for providing computer software for NMR data processing.

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BI962759M