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# Is the Binding of Magnesium(II) to Calmodulin Significant? An Investigation by Magnesium-25 Nuclear Magnetic Resonance†

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ABSTRACT: Previous reports on the interaction between calmodulin (CaM) and $Mg^{2+}$ range from no binding to a binding constant of $10^4$ $M^{-1}$ [for a summary, see Cox, J. A., Comte, M., Malnoe, A., Berger, D., & Stein, E. A. (1984) *Met. Ions Biol. Syst. 17*, 215–273]. In order to resolve the controversy, we used $^{25}Mg$ NMR to study the binding of $Mg^{2+}$ to apo-CaM, CaM·$Ca_2^{2+}$ (in which sites III and IV are occupied by $Ca^{2+}$), CaM·$La_2^{3+}$ (in which sites I and II are occupied by $La^{3+}$), and the two tryptic fragments of calmodulin, $TR_1C$ (containing sites I and II of CaM) and $TR_2C$ (containing sites III and IV of CaM). In each system, a "titration set" and a "temperature set" were obtained, and the spectral data were analyzed by total band-shape analysis to calculate the association constant ($K_a$) and off-rate ($k_{off}$). As in the case of $Ca^{2+}$ binding, sites I and II and sites III and IV were treated as two sets of equivalent sites, and a $Ca^{2+}/Mg^{2+}$ competition experiment suggested that $Mg^{2+}$ competes with $Ca^{2+}$ for the same sites. For both CaM·$Ca_2^{2+}$ and $TR_1C$, moderately large $K_a$ (2000 and 3500 $M^{-1}$, respectively) and moderate off-rates ($k_{off}$ = 2300 and 3000 $s^{-1}$, respectively, at 25 °C) were observed. For both CaM·$La_2^{3+}$ and $TR_2C$, binding of $Mg^{2+}$ was weaker by a factor of ca. 10 ($K_a$ = 300 and 200 $M^{-1}$, respectively) while the off-rates were also moderate ($k_{off}$ = 3500 and 2200 $s^{-1}$, respectively). In consistency with these, the data from binding studies of $Mg^{2+}$ to apo-CaM were fitted well with two strong sites ($K_a$ = 2000 $M^{-1}$, $k_{off}$ = 2700 $s^{-1}$ at 25 °C) and two weak sites ($K_a$ = 300 $M^{-1}$, $k_{off}$ = 6600 $s^{-1}$ at 25 °C). The results indicate that $Mg^{2+}$ shows opposite site preference relative to $Ca^{2+}$ and binds to sites I and II of CaM with a binding constant of ca. 2000 $M^{-1}$, as compared to ca. $5 \times 10^5$ $M^{-1}$ for $Ca^{2+}$. Since the intracellular concentration of $Mg^{2+}$ is higher than that of $Ca^{2+}$ by a factor of ca. $10^4$–$10^5$ at the resting state and ca. $10^2$–$10^3$ during stimulation of cells, it is possible that sites I and II are constantly occupied by $Mg^{2+}$ at the resting state and by $Mg^{2+}$ or $Ca^{2+}$ during stimulation. We hypothesize that sites I and II of CaM may indeed be "$Mg^{2+}$–$Ca^{2+}$ sites" and play a structural role in some systems, in analogy to sites III and IV of skeletal muscle troponin C [Johnson, J. D., Charlton, S. C., & Potter, J. D. (1979) *J. Biol. Chem. 254*, 3497–3502].

The biological functions of calmodulin (CaM)[1] [for recent reviews, see Manalan and Klee (1984) and Cox et al. (1984)] appear to be regulated by the binding of $Ca^{2+}$ ions to the four $Ca^{2+}$-binding domains, numbered I–IV starting from the N-terminus (Vanaman et al., 1977). The kinetics and thermodynamics of $Ca^{2+}$ binding to calmodulin have been studied extensively by various techniques and the results interpreted by various models [for recent reviews, see Potter et al. (1983), Cox et al. (1984), and Forsén et al. (1986)]. The model that has been gaining broad acceptance is that the four sites can be grouped into two high-affinity sites (sites III and IV) with binding constant $K_a \simeq 5 \times 10^6$ $M^{-1}$ and two low-affinity sites (sites I and II, $K_a \simeq 5 \times 10^5$ $M^{-1}$), with a certain degree of positive cooperativity between the two sites in each class (Wang, 1985). Such a model has been supported strongly by the results of $^{43}Ca$ and $^{113}Cd$ NMR studies (Drakenberg et al., 1983; Teleman et al., 1986; Andersson et al., 1982b; Thulin et al., 1984), $Tb^{3+}$-induced fluorescence studies (Kilhoffer et al., 1980a,b; Wallace et al., 1982; Wang et al., 1982, 1984), $Mn^{2+}$ paramagnetic resonance studies (Xu et al., 1983), $^1H$ NMR studies (Ikura et al., 1984), and stop-flow kinetics (Martin et al., 1985). The $^{43}Ca$ NMR and stop-flow kinetics gave $k_{off}$ values of 1150 and 650 $s^{-1}$, respectively, for the low-affinity sites and <40 and 9 $s^{-1}$, respectively, for the high-affinity sites (Drakenberg et al., 1983; Martin et al., 1985).

Considering the importance of $Ca^{2+}$–calmodulin complexes in the regulation of a wide variety of cellular events, it is surprising to find that the metal ion specificity of calmodulin (Chao et al., 1984; Wang et al., 1984) is much broader than that of phospholipase $A_2$, which has a considerably weaker affinity for $Ca^{2+}$ ($K_a = 4 \times 10^3$ $M^{-1}$) (Drakenberg et al., 1984). While substitution of $Ca^{2+}$ by $Cd^{2+}$ reduces the activity of phospholipase $A_2$ to <1% (Tsai et al., 1985), most cations with effective ionic radii in the range of 1 ± 0.2 Å can substitute for $Ca^{2+}$ (0.99 Å), both in binding and in the activation of calmodulin (Chao et al., 1984). The ubiquitous $Mg^{2+}$ ion, however, has an ionic radius of 0.65 Å and was thought not

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[1] Abbreviations: CaM, calmodulin; NMR, nuclear magnetic resonance; $TR_1C$, tryptic fragment of calmodulin containing residues 1–77; $TR_2C$, tryptic fragment of calmodulin containing residues 78–148; TnC, troponin C; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ATPase, adenosinetriphosphatase; cAMP, adenosine cyclic 3′,5′-monophosphate; $K_a$, association constant; $k_{off}$, off-rate of bound ion ($=1/\tau_B$); $\Delta G^{\ddagger}$, free energy of activation for $Mg^{2+}$ binding; $\Delta H^{\ddagger}$, enthalpy of activation for $Mg^{2+}$ binding; $\Delta S^{\ddagger}$, entropy of activation for $Mg^{2+}$ binding; $\chi_B$, quadrupolar coupling constant, $(e^2qQ/h)$, of bound $^{25}Mg^{2+}$; $R_{2B}$, transverse relaxation rate of bound $^{25}Mg^{2+}$; $\Delta\nu$, line width of $^{25}Mg^{2+}$ resonance; $\Delta\nu_B$, $\Delta\nu$ of bound $^{25}Mg^{2+}$; $\omega$, Larmor velocity; $\tau_c$, correlation time; $Q$, quadrupole moment; $P_A$ and $P_B$, molar fraction of free and bound ions, respectively.

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to bind or activate calmodulin, according to Chao et al. (1984) and others (Cox et al., 1981; Dedman et al., 1977).

Some researchers, however, have demonstrated binding of $Mg^{2+}$ to calmodulin. The most convincing evidence came from $^1H$ NMR studies, which indicated chemical shift changes induced by $Mg^{2+}$, though different from the changes induced by $Ca^{2+}$ (Seamon, 1980; Sutoo et al., 1986). Shimizu and Hatano (1985) also showed that the $^{25}Mg$ NMR signal of $Mg^{2+}$ was broadened upon addition of up to 10 mol % of calmodulin and that such a broadening was reversed upon addition of $Ca^{2+}$. Several other reports employing equilibrium dialysis or $Ca^{2+}$ electrode have shown that $Mg^{2+}$ can affect the binding constants of $Ca^{2+}$ to calmodulin appreciably, and binding constants ranging from $10^2$ to $10^4$ $M^{-1}$ have been obtained for $Mg^{2+}$ (Crouch & Klee, 1980; Haiech et al., 1981; Potter et al., 1981; Wolff et al., 1977; Ogawa & Tanokura, 1984; Iida & Potter, 1986).

Although the various reports cited above deviate greatly from one another, they seem to suggest that $Mg^{2+}$ binds to calmodulin, possibly to the calcium sites, with a binding constant $K_a$ in the order of $10^3$ $M^{-1}$. A key question not addressed in previous studies is the relative affinity of $Mg^{2+}$ to the two classes of $Ca^{2+}$ sites. Since the intracellular concentration of $Mg^{2+}$ is in the order of $10^{-3}$ M (Williams, 1980; Gupta et al., 1978) and that of $Ca^{2+}$ is $\sim 10^{-7}$ M in the resting state and increases to $10^{-6}$–$10^{-5}$ M during stimulation (Williams, 1980), binding of $Mg^{2+}$ may compete favorably with binding of $Ca^{2+}$ at the weaker $Ca^{2+}$ sites (I and II), if the binding constants observed for $Mg^{2+}$ arise from binding of $Mg^{2+}$ to sites I and II. Our favorable experience in using quadrupolar $^{43}Ca$ NMR ($I = {}^7/_2$; $Q = -0.05 \times 10^{-24}$ $cm^2$) (Olsson & Salomonson, 1982; Grundvik et al., 1979) to establish the site preference of $Ca^{2+}$ binding to calmodulin and its proteolytic fragments $TR_1C$ and $TR_2C$ (Drakenberg et al., 1983; Teleman et al., 1986) prompted us to investigate the kinetic and thermodynamic properties of $Mg^{2+}$ binding to calmodulin using $^{25}Mg$ NMR ($I = {}^5/_2$, $Q = 0.22 \times 10^{-24}$ $cm^2$) (Lutz et al., 1975). The experimental approaches and data analysis parallel those of $^{43}Ca$ NMR (Drakenberg et al., 1983), but $^{25}Mg$ NMR is technically more difficult due to the larger quadrupole moment of $^{25}Mg$ relative to that of $^{43}Ca$.

## Materials and Methods

*Materials.* Bovine testis calmodulin (CaM) and its two tryptic fragments, $TR_1C$ and $TR_2C$, were prepared, and their purity was checked as previously described (Andersson et al., 1983; Vogel et al., 1983). Absence of EDTA in these samples was confirmed by $^1H$ NMR at 360 MHz. $^{25}MgO$ (98 atom % $^{25}Mg$) was purchased from Oak Ridge National Laboratory. The $^{25}Mg^{2+}$ stock solution (0.1 M) was prepared by dissolving $^{25}MgO$ in 0.4 M $HClO_4$ and adjusted to pH 8.0 with NaOH. All other chemicals used were of reagent grade.

*$^{25}Mg$ NMR Experiments.* The $^{25}Mg$ NMR spectra were recorded at 22.15 MHz on a Nicolet NT-360 wide-bore NMR spectrometer. The probe used was built in our laboratory (Drakenberg et al., 1983) and possesses a solenoid coil. The horizontal sample tube used is 17 mm in diameter and holds 3.0 mL of liquid. The spectra were obtained unlocked and without sample spinning. The standard 1 M $MgCl_2$ solution (pH 8.0), which gives a sharp signal (line width $\Delta\nu < 5$ Hz) under high-resolution conditions [Lindman et al. (1977) and references cited therein], gave $\Delta\nu = 35$ Hz (including 10-Hz line broadening) under our experimental conditions at 25 °C. Such a contribution by field inhomogeneity (ca. 20 Hz) was small compared to most of the observed $\Delta\nu$ and was not taken into consideration in data analysis. In order to minimize the effects of probe ringing, we used a special pulse sequence (Vogel & Forsen, 1986; P. D. Ellis, private communication in 1982) to cancel the effect of acoustic ringing and introduced two left shifts to eliminate the first two data points prior to Fourier transformation. The number of transients varied from 50 000 to 3 000 000 depending on the concentration and the line width and was collected in blocks of 5000 transients and then block averaged in double precision. Typically, the 90° pulse was 40 μs, the acquisition time was 25.6 ms (for broad signals) or 51.2 ms (for narrow signals), the spectral width was ±20 000 or ±10 000 Hz, and the preacquisition delay was 25 or 50 μs. A line broadening of 50, 100, or 200 Hz was applied to different sets of experiments.

Typically, two sets of experiments were performed for each protein system: a "titration set" at 25 °C (with successive addition of $Mg^{2+}$ to a ca. 1 mM protein solution) and a "temperature set" (with temperature variation from 1 to 65 °C, at a fixed ratio of $[Mg^{2+}]/[protein]$). The sample was prepared by dissolving the protein (typically 3 μmol) in 3.0 mL of $H_2O$ (pH ca. 10), followed by addition of the desired metal ions ($CaCl_2$ or $LaCl_3$) and the first aliquot of a 0.1 M $^{25}Mg^{2+}$ solution (typically 20 μL) and then with addition of 0.05 M HCl until the pH reached 8.0. After each further addition of $^{25}Mg^{2+}$, the pH was adjusted back to 8.0 with 0.05 M NaOH. At the later part of titration an unenriched 1 M $MgCl_2$ solution was used. Although it would be preferable to add other ions such as $Na^+$ or $K^+$ to mimic the physiological condition, we chose not to do so in order to avoid possible complications in the interpretation of $^{25}Mg$ NMR data. As mentioned under Discussion, the effect of $K^+$ will be investigated in the future.

The concentrations of the stock solutions of the cations used were determined by atomic absorption. The apocalmodulin and its tryptic fragments usually contain 0.1–0.4 and 0.05–0.1 equiv, respectively, of $Ca^{2+}$ as indicated by atomic absorption. In the $Ca^{2+}/Mg^{2+}$ competition experiment and in the $Mg^{2+}$ titration of $CaM{\cdot}Ca_2^{2+}$, the residual $Ca^{2+}$ content was included in the total concentration of $[Ca^{2+}]$, while in other experiments it was not corrected in data analysis. The protein concentration was first determined on the basis of the initial total weight and the total volume at each titration point and corrected by applying a "correction factor" of 0.8–0.9 in calculations to obtain the best fitting. The protein concentrations in the figures are the corrected values. Routinely, the protein concentrations were also determined by UV absorbance (Newton et al., 1984) at the end of the NMR experiments (the metal ions were found to have very small effects on the extinction coefficients of CaM), and the values often agree with those obtained by data fitting. After the temperature experiments, a spectrum at 25 °C was obtained to assess possible denaturation. For calmodulin the same $\Delta\nu$ was obtained before and after heating, and the protein was recovered for reusage by extensive dialysis followed by passage through a column of Chelex-100 (washed extensively with $H_2O$ followed with dilute $NH_4OH$, pH 8, before usage). For $TR_1C$ and $TR_2C$ some denaturation occurred after heating, and the protein was not reutilized.

*Data Analysis.* Using the case of $Mg^{2+}$ binding to $CaM{\cdot}Ca_2^{2+}$ as an example (see the first section under Results), we describe the steps involved and the assumptions made in the total line-shape analysis. The general principles have been described and illustrated by several examples for $^{43}Ca$ NMR (Drakenberg et al., 1983), and we only emphasize the practical aspects relevant to the present systems. The observed spectra in Figure 1 indicate that the NMR signal of $Mg^{2+}$ is appreciably broadened by CaM and suggest a significant affinity

of $Mg^{2+}$ to CaM. The line shapes are approximately Lorentzian within experimental errors, which suggests two possibilities: very fast exchange (the observed signals are due to average of free and bound $Mg^{2+}$) or slow exchange (the observed signals are predominantly due to the free $Mg^{2+}$ broadened by exchange; the signal of the bound $Mg^{2+}$ is likely to be broadened beyond the limit of detection; the situation is to be differentiated from "*very* slow exchange" where the signal of free $Mg^{2+}$ should not be broadened by exchange). The temperature curve in Figure 2B rules out the first possibility since it indicates the exchange rate goes from "slow" via "intermediate" to "fast" with increasing temperature. Thus, it should be possible to determine the exchange rate from the temperature curve. In addition, since the titration curve (Figure 2A) does not show a clear turning point at $[Mg^{2+}]/[\text{protein}] = 2.0$, the binding constant should also be in the measurable range. We then set forth to perform the total band-shape analysis as summarized below (Drakenberg et al., 1983):

(1) Bloch equations modified to take into account the effect of exchange (McConnell, 1958) were used to derive a band-shape equation for the exchange system. The parameters required to define such a band-shape are as follows (A and B refer to free and bound ions, respectively):

(*a*) *Chemical Shifts* $\delta_A$ *and* $\delta_B$. Values of 0 and 20 Hz, respectively, were used. The $\delta_B$ value was probably not accurate, but it should have little effect on the calculations since $\Delta\delta$ is quite small compared to the line width.

(*b*) *Populations* $P_A$ *and* $P_B$. These are defined by $[Mg^{2+}]$, [protein] (experimental values), and binding constant $K_a$. In all calculations sites I and II were treated as two equivalent sites, as were sites III and IV.[2]

(*c*) *Transverse Relaxation Rates* $R_{2A}$ $(=1/T_{2A})$ *and* $R_{2B}$ $(=1/T_{2B})$. In the present systems, $R_{2A}$ is negligible relative to $R_{2B}$ in the absence of exchange. According to Halle and Wennerstrom (1981), for nuclei with $I = {}^5/_2$ or ${}^7/_2$ and for $\omega\tau_c < 1.5$, $R_{2B}$ can be described by

$$R_{2B} = \frac{3\pi^2}{10}\chi_B{}^2\frac{2I+3}{I^2(2I-1)}\left[0.3\tau_c + \frac{0.5\tau_c}{1+(\omega\tau_c)^2} + \frac{0.2\tau_c}{1+(2\omega\tau_c)^2}\right] \quad (1)$$

where $\chi_B$ is the quadrupolar coupling constant for the bound $^{25}Mg^{2+}$.

(2) The $\tau_c$ values for the bound $^{25}Mg^{2+}$ were assumed to be the same as those determined from $^{43}Ca$ NMR: 8.2 ns for calmodulin (Andersson et al., 1982a) and 6.0 ns for $TR_2C$ (Teleman et al., 1986) (which is also assumed for $TR_1C$). In other words, it is assumed that both $Mg^{2+}$ and $Ca^{2+}$ are bound rigidly, such that the $\tau_c$ for the bound ion approximates the $\tau_c$ of the protein itself. If the bound $Mg^{2+}$ possesses internal rotational freedom relative to the protein, $\chi_B$ will be underestimated, but $k_{off}$ at 25 °C and binding constant $K_a$ will not be affected. The temperature dependence of $\tau_c$ is assumed to be defined by the transition state type equation:

$$\tau_c^{-1} = \frac{kT}{h}\exp(-\Delta G/RT) \quad (2)$$

The $\Delta G$ values used in the analysis were calculated from the $\tau_c$ values at 25 °C: 6418 cal $mol^{-1}$ for CaM and 6232 cal $mol^{-1}$ for its fragments. The $\omega\tau_c$ obtained for CaM at 25 °C is 1.14, which justifies the use of eq 1. At lower temperatures, $\omega\tau_c$ may exceed 1.5 (e.g., $\omega\tau_c = 2.67$ at 1 °C), but we believe the errors resulting from deviation of eq 1 will still be quite small. In particular, the $k_{off}$ under such a slow exchange condition is mainly deduced from the $\Delta\nu$ of the "free" $Mg^{2+}$ while eq 1 concerns only the $\Delta\nu$ of "bound" $Mg^{2+}$.

(3) The temperature dependence of $k_{off}$ is also assumed to follow the transition-state theory:

$$k_{off} = \frac{kT}{h}e^{-\Delta G^*/RT} = \frac{kT}{h}e^{[(\Delta S^*/R)-(\Delta H^*/RT)]} \quad (3)$$

(4) The association constant $K_a$ is assumed to be temperature independent. This may give substantial errors in the fitting of the temperature curve of the weaker sites since they are only 50–70% saturated under the present experimental conditions, thus sensitive to temperature variations in $K_a$. This, however, will not affect the conclusion of this work as discussed later.

(5) Digitized NMR spectra, defined by 50 points each, were then used in an iterative least-squares fit to find a proper combination of values for $\Delta S^*$ and $\Delta H^*$ (which define $k_{off}$), $\chi_B$, and $K_a$. In general, $k_{off}$ is best defined by the "slow" region of the temperature curve, $\chi_B$ by the "intermediate" and "fast" regions of the temperature curve, and $K_a$ by the titration curve. However, all the parameters are interrelated, and the set of values that fit *both* curves are accurate to within 5% in the analysis (i.e., by changing one of the parameters by 5% a poor fit will be obtained). The more important question is how accurately do these parameters represent the "real" values. Taking into consideration the possible errors in concentrations, temperatures, field inhomogeneity, signal/noise ratio, and the possibility of fitting at a local minimum, we estimate that the good data sets (those for sites I and II, with complete temperature and titration curves) should be accurate to within ±20% in $K_a$, $k_{off}$, and $\chi_B$. The $\Delta S^*$ may have larger errors, and the physical meaning for the strongly negative $\Delta S^*$ values is not clear. However, this should not affect other parameters and conclusions.

(6) In order to show the agreement between the experimental spectra and the calculated spectra (defined by the best fit parameters at each experimental condition), we plot the line widths of both experimental and calculated spectra as a function of [metal]/[protein] ratio and temperature. Two points need clarification: (i) although the *x* axis of the titration plot is the [metal]/[protein] ratio, the actual concentrations, which varied slightly from point to point, were used in the iterative fitting; (ii) although the $\Delta\nu$ are obtained by Lorentzian fitting of the experimental or calculated spectra, the "total line-shape analysis" does not assume a Lorentzian line shape. The plots of $\Delta\nu$ are used only to provide a visual comparison and to avoid the necessity to show every experimental and calculated spectrum. All spectra happen to be very close to Lorentzian under the experimental conditions of this work, as shown in Figure 1.

## Results

*$Mg^{2+}$ Binding to Sites I and II of CaM.* It is technically simpler and less ambiguous to first examine the binding of $Mg^{2+}$ to each of the two classes of binding sites separately. Since under our experimental conditions $Ca^{2+}$ binds to sites III and IV with a higher affinity than to sites I and II by a factor of 10–100 (Andersson et al., 1983; Forsén et al., 1983a; Wang, 1985), we have been able to study the binding properties of $Mg^{2+}$ to sites I and II using $CaM{\cdot}Ca_2^{2+}$. Figure 1

[2] We have also considered the possibility of other weak $Mg^{2+}$ sites in the line-shape analysis. As shown in Table I, one of the data sets can only be fitted with the introduction of two additional weak $Mg^{2+}$ sites. The significance of this result is unclear.

Table I: Summary of Kinetic, Thermodynamic, and Spectral Parameters for the Binding of $Mg^{2+}$ to Calmodulin and Its Tryptic Fragments, at 25 °C, pH 8.0

| system | sites | $K_a$ ($M^{-1}$)[a] | $k_{off}$ ($s^{-1}$)[a] | $\Delta G^{\ddagger}$ (kcal/mol) | $\Delta H^{\ddagger}$ (kcal/mol)[a] | $\Delta S^{\ddagger}$ [cal/(mol·K)][a] | $\chi_B$ (MHz)[a] | $R_{2B}$ (kHz) | $\Delta\nu_B$ (kHz) |
|---|---|---|---|---|---|---|---|---|---|
| CaM·$Ca_2^{2+}$[b] | I, II | 2000 | 2300 | 12.87 | 8.7 | −14 | 1.6 | 10.9 | 3.5 |
| $TR_1C$ | I, II | 3500 | 3000 | 12.70 | 5.3 | −25 | 1.5 | 8.2 | 2.6 |
| apo-CaM | I, II | 2000 | 2700 | 12.77 | 8.3 | −15 | 1.7 | 12.5 | 4.0 |
| CaM·$La_2^{3+}$ | III, IV | 300 | 3500 | 12.61 | 2.2 | −35 | 1.5 | 10.3 | 3.3 |
| $TR_2C$ | III, IV | 200 | 2200 | 12.88 | 2.8 | −34 | 1.5 | 8.2 | 2.6 |
| apo-CaM | III, IV | 300 | 6600 | 12.24 | 2.7 | −32 | 1.7 | 12.5 | 4.0 |

[a] The values for these parameters were obtained directly from the iterative fitting described under Data Analysis. [b] In this particular calculation, the best fit was obtained with the introduction of two weak nonspecific $Mg^{2+}$ sites with $K_a$ = 27 $M^{-1}$ and $\Delta G^{\ddagger}$ = 3.6 kcal/mol.

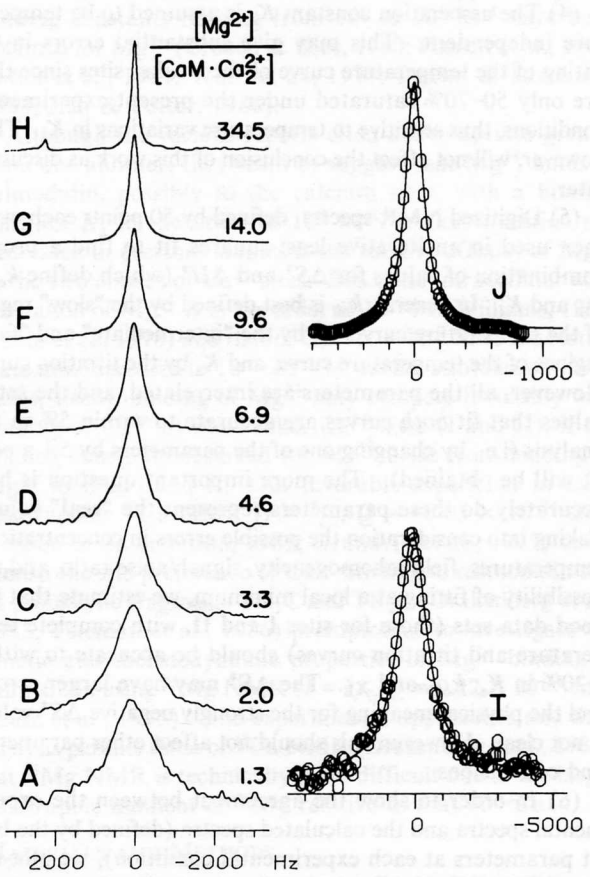


FIGURE 1: $^{25}Mg$ NMR spectra (at 22.15 MHz, 25 °C) of $Mg^{2+}$ at various ratios of [$Mg^{2+}$]/[CaM·$Ca_2^{2+}$] (A–H), which correspond to half of the experimental points (every other point) in Figure 2A. The detailed conditions are described under Materials and Methods. Spectra I and J are the Lorentzian fitting of spectra A and H, respectively. The circles in spectra I and J represent experimental data, and the solid lines represent the fitted spectra. The line broadening used is 100 Hz.

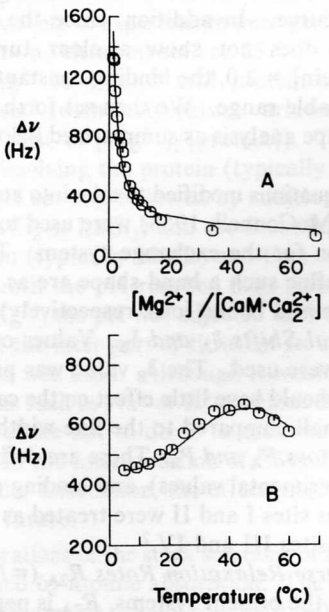


FIGURE 2: Titration curve (A) and temperature curve (B) of $Mg^{2+}$ binding to CaM·$Ca_2^{2+}$. The titration curve was obtained at 25 °C, with [CaM·$Ca_2^{2+}$] varying from 0.98 mM at the first point to 0.78 mM at the last point. The temperature curve was obtained at [$Mg^{2+}$]/[CaM·$Ca_2^{2+}$] = 5.24. Circles are experimental points, and solid curves are obtained by connecting the calculated points from the iterative fitting. The $\Delta\nu$ shown include a line broadening of 100 Hz.

shows some of the $^{25}Mg$ NMR spectra at different ratios of [$Mg^{2+}$]/[CaM·$Ca_2^{2+}$]. The line widths ($\Delta\nu$) of the $^{25}Mg$ NMR signals are plotted as a function of the [$Mg^{2+}$]/[CaM·$Ca_2^{2+}$] ratios in Figure 2A. The temperature dependence of $\Delta\nu$ at [$Mg^{2+}$]/[CaM·$Ca_2^{2+}$] = 5.24 is plotted in Figure 2B. The solid curves were obtained by fitting the spectra with total line-shape analysis (Drakenberg et al., 1983). The kinetic and thermodynamic parameters obtained are summarized in Table I.[2]

It is obvious from the titration curve (Figure 2A) and the temperature dependence curve (Figure 2B) that the $^{25}Mg$ NMR signal is substantially broadened upon binding to sites I and II of CaM and that the exchange rate goes from "slow" via "intermediate" to "fast" with increasing temperature. Such a behavior is similar to the $^{43}Ca$ NMR properties of $Ca^{2+}$ binding to sites I and II of CaM (Drakenberg et al., 1983). The binding constant of $Mg^{2+}$ is smaller than that of $Ca^{2+}$ by 2 orders of magnitude, but binding of $Mg^{2+}$ to sites I and II of CaM could still be physiologically significant since the physiological concentration of $Mg^{2+}$ is higher than that of $Ca^{2+}$ by ca. $10^4$ in the resting state (Williams, 1980). This point will be further elaborated under Discussion.

It should be noted that although [$Mg^{2+}$] is less than the concentration of binding sites in Figure 1A, the observed signal ($\Delta\nu$ = 1400 Hz) is not due to bound $Mg^{2+}$. On the basis of the binding constant and the concentrations used, 32% of $Mg^{2+}$ is not bound and gives rise to the exchange-broadened signal. On the basis of eq 1 and the best fit parameters at 25 °C, the line width of the signal of the bound $Mg^{2+}$ should be 3500 Hz (last column in Table I), which is beyond the limit of detection under the experimental conditions. Spectrum 1A was obtained with 2.4 million transients in 18 h, and the signal/noise ratio of the free $Mg^{2+}$ signal is only ca. 10. An increase in $\Delta\nu$ by a factor of 2.5 will result in loss of signal due to smaller signal/noise ratio and, in particular, loss of information during the "dead time".

*$Mg^{2+}$ Binding to the N-Terminal Tryptic Fragment of CaM ($TR_1C$).* It has been shown previously that $TR_1C$ consists of sites I and II of CaM and behaves very similarly to the native

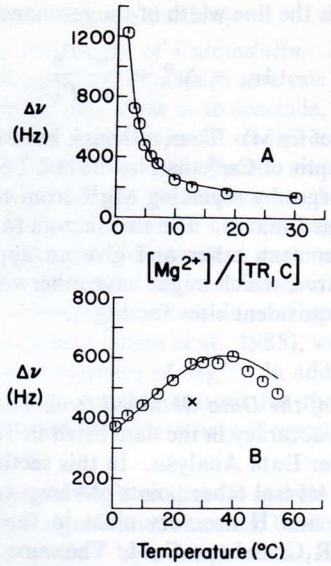


FIGURE 3: Titration curve (A) and temperature curve (B) of $Mg^{2+}$ binding to $TR_1C$. The titration curve was obtained at 25 °C, with $[TR_1C]$ varying from 0.54 mM at the first point to 0.51 mM at the last point. The temperature curve was obtained at $[TR_1C] = 0.57$ mM and $[Mg^{2+}] = 2.32$ mM. Circles are experimental points, and solid curves are obtained by connecting the calculated points from the iterative fitting. The extra point (×) at 26 °C in the temperature curve was obtained after heating and indicated partial denaturation of the protein. Thus, the last three points (45, 50, and 55 °C) were not used in the iterative fitting. The $\Delta\nu$ shown include a line broadening of 100 Hz.

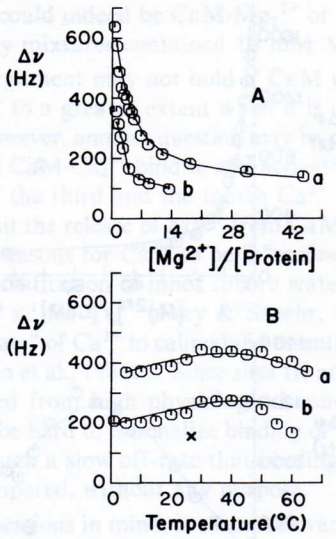


FIGURE 4: Titration curves (A) and temperature curves (B) of $Mg^{2+}$ binding to $CaM{\cdot}La_2^{3+}$ (curve a, the upper curve in both A and B) and to $TR_2C$ (curve b, the lower curve in both A and B). The titration curves were obtained at 25 °C. Circles are experimental points, and solid curves are obtained by connecting the calculated points from the iterative fitting. For $CaM{\cdot}La_2^{3+}$ (curves a), the starting and ending $[CaM{\cdot}La_2^{3+}]$ were 1.12 and 0.96 mM, respectively, the temperature curve was obtained at $[Mg^{2+}]/[CaM{\cdot}La_2^{3+}] = 2.85$, and the $\Delta\nu$ include a line broadening of 100 Hz. For $TR_2C$ (curves b), the starting and ending $[TR_2C]$ were 1.22 and 0.70 mM, respectively, the temperature curve was obtained at $[Mg^{2+}]/[TR_2C] = 2.12$, and the $\Delta\nu$ include a line broadening of 50 Hz. The extra point (×) at 25 °C in temperature curve b for $TR_2C$ was obtained after heating and indicated partial denaturation of the protein. Thus, the last three points (50, 55, and 60 °C) were not used in the iterative fitting for $TR_2C$.

CaM in terms of binding to $Ca^{2+}$ (Teleman et al., 1986) and to $Cd^{2+}$ (Andersson et al., 1983; Thulin et al., 1984), although it does not possess the full biological functions of the native CaM. We therefore used $TR_1C$ to further confirm the binding of $Mg^{2+}$ to sites I and II of CaM. The titration curve and the temperature dependence in $\Delta\nu$ are plotted in Figure 3 (A and B, respectively) and fitted with the total line-shape analysis. The relatively sharp decrease in $\Delta\nu$ at higher temperatures was found to be caused by partial denaturation of the protein, since the control run at 26 °C after heating showed a ca. 50% reduction in $\Delta\nu$. Thus, only the points at 40 °C and lower were used in the iterative fitting, and the theoretical curve on the high-temperature side was calculated from the best fit parameters listed in Table I. The results are in the same orders of magnitudes as those of $CaM{\cdot}Ca_2^{2+}$.

*$Mg^{2+}$ Binding to Sites III and IV of CaM.* It has been demonstrated by luminescence studies (Wang et al., 1982, 1984; Kilhoffer et al., 1980a,b; Mulqueen et al., 1985; Wallace et al., 1982) that lanthanide ions such as $Tb^{3+}$ and $Dy^{3+}$ preferentially bind to sites I and II of CaM. The binding constants for $Tb^{3+}$ were estimated to be $10^7$–$10^8$ $M^{-1}$ and $1.5 \times 10^5$ $M^{-1}$ for the high-affinity sites (I, II) and the low-affinity sites (III, IV), respectively (Wang et al., 1984). Using $^1H$ NMR, Teleman (1986) has also show that addition of 2 equiv of $La^{3+}$ or $Lu^{3+}$ induces shifts of a group of signals corresponding to the shifts induced by $Ca^{2+}$ at the second half of the $Ca^{2+}$ titration. Thus, $CaM{\cdot}Ln_2^{3+}$ can be used as a model to study the interaction of $Mg^{2+}$ with sites III and IV of CaM, and we chose the diamagnetic $La^{3+}$ to avoid complications by paramagnetic effects.

The titration curve in Figure 4A (curve a) indicates that binding of $Mg^{2+}$ to $CaM{\cdot}La_2^{3+}$ is much weaker than the corresponding binding to $CaM{\cdot}Ca_2^{2+}$. The temperature curve (Figure 4B, curve a), however, indicates that the exchange rate is still in the intermediate range at room temperature. Since the temperature dependence in $\Delta\nu$ is smaller in this case, the experimental points do not show continual changes smoothly. Nonetheless, there is a clear maximum, and both curves can be fitted with parameters comparable to those of sites I and II except with a ca. 10-fold reduction in $K_a$ as also listed in Table I.

*$Mg^{2+}$ Binding to the C-Terminal Tryptic Fragment of CaM ($TR_2C$).* The weak binding of $Mg^{2+}$ to sites III and IV of CaM was also confirmed with the C-terminal tryptic fragment $TR_2C$, as shown by the titration curve in Figure 4A (curve b). The temperature dependence curve in Figure 4B (curve b) also indicates intermediate exchange at room temperature. However, the sharp decrease in $\Delta\nu$ at higher temperatures was again found to be caused by partial denaturation of $TR_2C$ (even though no precipitate formed), since the $\Delta\nu$ at 25 °C was found to decrease from 230 Hz prior to the temperature study to 165 Hz after heating to 60 °C. Since there are not enough data to define $\chi_B$, we used the same $\chi_B$ as in $TR_1C$ (1.5 MHz) and obtained values of $k_{off}$ and $K_a$ comparable to those of $CaM{\cdot}La_2^{3+}$, as shown in Table I.

*$Mg^{2+}$ Binding to Apo-CaM.* The titration curve (Figure 5A) and the temperature curve (Figure 5B) can be fitted with two strong sites and two weak sites, assuming the same $\chi_B$ in both sites. On the basis of the larger binding constants in $CaM{\cdot}Ca_2^{2+}$ and $TR_1C$, compared to those of $CaM{\cdot}La_2^{3+}$ and $TR_2C$, it is most reasonable to assign the strong sites to sites I and II and the weak sites to sites III and IV in the binding of $Mg^{2+}$ to apo-CaM. The results (Table I) show that $\chi_B$ (1.7 MHz) and $K_a$ (2000 and 300 $M^{-1}$) agree well with the other systems with isolated sites (note: they are not expected to be identical). The $k_{off}$ for sites III and IV of apo-CaM (6600 $s^{-1}$) is higher than that of $CaM{\cdot}La_2^{3+}$ and $TR_2C$ by a factor of 2–3, but we noted earlier that the temperature curves in the latter two systems were quite rough. In addition, we noted under Data Analysis that the assumption of a temperature-

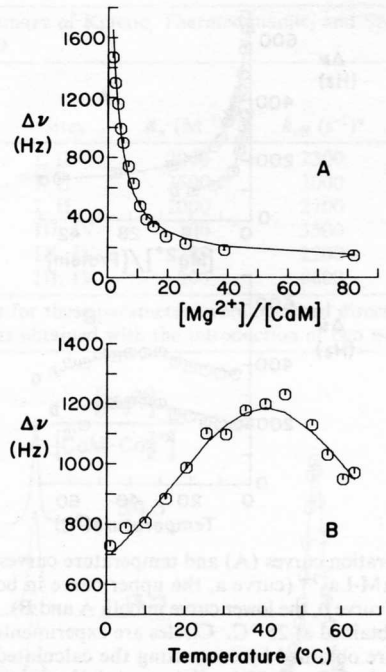


FIGURE 5: Titration curve (A) and temperature curve (B) of $Mg^{2+}$ binding to apo-CaM. The titration curve was obtained at 25 °C, with [CaM] varying from 0.75 to 0.65 mM. The temperature curve was obtained at [CaM] = 1.0 mM and [$Mg^{2+}$] = 5.0 mM. Circles are experimental points, and solid curves are obtained by connecting the calculated points from iterative fitting. The $\Delta\nu$ include a line broadening of 100 Hz in (A) and 200 Hz in (B).

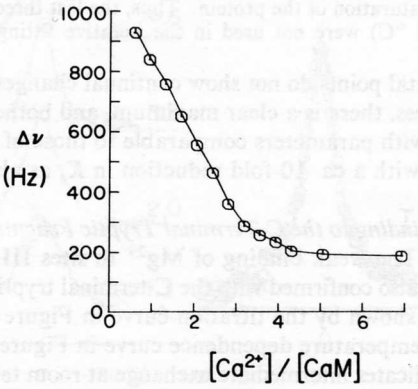


FIGURE 6: Addition of $CaCl_2$ to $Mg^{2+}$-CaM to show competition between $Ca^{2+}$ and $Mg^{2+}$. The experiment was performed at 25 °C, pH 7.9, with [CaM] = 0.95 mM, [$Mg^{2+}$] = 5.0 mM, and varying ratios of [$Ca^{2+}$]/[CaM]. The $\Delta\nu$ include a line broadening of 100 Hz.

independent binding constant may give rise to some error in the temperature curve, particularly to weak sites. Thus, the difference of a factor of 2–3 should not be taken seriously.

Figure 6 shows the result of a competition experiment between $Ca^{2+}$ and $Mg^{2+}$. Addition of ca. 4 equiv of $Ca^{2+}$ (relative to CaM) completely reversed the CaM-induced broadening in the $^{25}Mg^{2+}$ NMR signal. This justifies our assumption that $Mg^{2+}$ binds to calcium sites in CaM. Quantitative fitting of the competition curve is beyond our ability at this time. However, it may be questioned why the curve is apparently linear at lower ratios of [$Ca^{2+}$]/[CaM], if the first 2 equiv of $Ca^{2+}$ (which bind to the strong calcium sites, III and IV) compete with the weaker binding of $Mg^{2+}$. This can be explained qualitatively by $k_{off}$ values. As shown in Table I, the $k_{off}$ of 6600 for sites III and IV is closer to the relaxation rate ($R_{2B}$) of the bound $Mg^{2+}$ but is still in the slow exchange side. The line width of the resonance of the free (exchanging) $Mg^{2+}$, $\Delta\nu_A$, under this condition is given by eq 4 (where $\Delta\nu_A^0$ is the line width of the resonance of free $Mg^{2+}$ in the absence of CaM). Thus, although less $Mg^{2+}$ is replaced by the first 2 equiv of $Ca^{2+}$ than by the last 2 equiv, the effect on the $\Delta\nu$ is larger by replacing $Mg^{2+}$ from sites III and IV than from sittes I and II. The two factors ($k_{off}$ and $P_B/P_A$) compensate for each other and give an apparently linear competition curve, which might have otherwise been used to suggest four equivalent sites for $Mg^{2+}$.

$$\Delta\nu_A = \Delta\nu_A^0 + \frac{k_{off}}{\pi}\frac{P_B}{P_A} \quad (4)$$

## Discussion

*Evaluation of the Data Obtained from Total Line-Shape Analysis.* The accuracy in the data listed in Table I has been addressed under Data Analysis. In this section we evaluate the data from several other points of view. (a) $K_a$, $k_{off}$, and $\chi_B$ for sites I and II are very close in the three systems CaM·$Ca_2^{2+}$, $TR_1C$, and apo-CaM. The same is true for sites III and IV. These are in good agreement with the independence in the metal ion binding properties between the two classes of binding sites revealed previously in $^{43}Ca$ NMR and $^{113}Cd$ NMR studies (Andersson et al., 1982b; Thulin et al., 1984; Teleman et al., 1986). (b) The $\chi_B$ values seem to be smaller than expected. $^{43}Ca$ NMR studies gave $\chi_B$ = 1.08 MHz for $Ca^{2+}$ bound to CaM and its tryptic fragments (Drakenberg et al., 1983; Teleman et al., 1986). Thus, in the bound state the $\chi_B$ of $Mg^{2+}$ is greater than that of $Ca^{2+}$ by ca. 50%. The difference between the $\chi$ values of free $Mg^{2+}$ and free $Ca^{2+}$ is significantly larger. The $T_1$ values for a 1 M $MgCl_2$ solution and a 0.2 M $CaCl_2$ solution have been found to be 0.192 and 1.33 s, respectively (Lindman et al., 1977). At the same concentration the $T_1$ of $Ca^{2+}$ is ca. 5 times as long as the $T_1$ of $Mg^{2+}$. The relationship between $T_1$ and $\chi$ for small ions is governed by

$$\frac{1}{T_1} = \frac{3\pi^2}{10}\chi^2\frac{2I+3}{I^2(2I-1)}\tau_c \quad (5)$$

If $\tau_c$ is assumed to be the same, $(\chi_{Mg}/\chi_{Ca})^2$ should be ca. 12. By such a rough estimation, $\chi_{Mg}/\chi_{Ca}$ should be ca. 3.5, and the $\chi_B$ of $Mg^{2+}$ would be ca. 3.8 MHz if the ratio remains the same in the bound state. Two possible reasons for the "smaller than expected" $\chi_B$ of $Mg^{2+}$ are a more symmetrical environment and an internal rotational freedom (i.e., less rigid binding) for the bound $Mg^{2+}$ ion. (c) The on-rate of $Mg^{2+}$ ($k_{on} \simeq K_a k_{off}$) is ca. (0.5–1.0) × $10^7$ $s^{-1}$ $M^{-1}$ for sites I and II and ca. (0.5–2.0) × $10^6$ $s^{-1}$ $M^{-1}$ for sites III and IV. Thus, the larger binding constant of sites I and II is mainly caused by a larger on-rate. The $k_{on}$ of $Mg^{2+}$ thus obtained seem quite large, but they are within the upper limit given by $k_{is}K_{os}$ according to the Eigen–Tamm mechanism (Eigen & Tamm, 1962), where $k_{is}$ is the first-order rate constant of dehydration in the inner solvation sphere, usually ~$10^5$ $s^{-1}$ for $Mg^{2+}$ (Eigen, 1963), and $K_{os}$ is the equilibrium constant for the formation of an outer sphere complex between the solvated metal ion and the ligands of the complexation agent. The $k_{on}$ of MgATP (1.3 × $10^7$ $s^{-1}$ $M^{-1}$) is among the highest values reported (Diebler et al., 1969). The $k_{off}$ and $k_{on}$ of $Mg^{2+}$ binding to troponin C have been estimated as 4 × $10^3$ $s^{-1}$ and 1.6 × $10^7$ $s^{-1}$, respectively, from $^{25}Mg$ NMR studies using a large excess of $Mg^{2+}$ relative to TnC (Forsén et al., 1983a). Although NMR is not the most accurate technique to measure on- and off-rates, there is yet no ideal $Mg^{2+}$ chelating agent that carries a fluorescent chromophore and binds $Mg^{2+}$ rapidly (>$k_{on}$ of $Mg^{2+}$ to CaM) and tightly (1–2 orders of magnitude higher

in binding constant than the protein).

*$Mg^{2+}$ Binding Properties of Calmodulin.* Although the $^{25}$Mg NMR technique and line-shape analysis have implicit errors in quantitation, they allow us to conclude, undisputably, even with only visual examination of the spectral data, that $Mg^{2+}$ does bind to CaM, but with opposite site preference to $Ca^{2+}$. Such a reversed site preference has been observed previously for trivalent lanthanide ions (Wang et al., 1984; Teleman, 1986) but not for divalent cations. Since the structural difference between the two classes of $Ca^{2+}$ binding sites is not obvious even in the three-dimensional structure of CaM (at 3-Å resolution) (Babu et al., 1985), we are not able to explain the site preference of $Mg^{2+}$. In addition, we will not attempt to assess possible reasons for the discrepancy between our results and the wide variety of reports summarized in the introduction.

The weak binding of $Mg^{2+}$ to sites III and IV may not have much physiological significance since the binding constant is smaller than that of $Ca^{2+}$ by a factor of ca. $10^5$. However, binding of $Mg^{2+}$ to apo-CaM and to CaM·$Ca_2^{2+}$ could be very important. The binding constant of $Ca^{2+}$ to CaM·$Ca_2^{2+}$ is ca. $5 \times 10^5$ $M^{-1}$ (Wang, 1985), greater than that of $Mg^{2+}$ by ca. $10^2$. The intracellular $Ca^{2+}$ concentration is $<10^{-7}$ M at resting state and increases to $10^{-6}$–$10^{-5}$ M upon stimulation, while that of $Mg^{2+}$ is in the order of $10^{-3}$ M (Williams, 1980; Gupta et al., 1978). Although $Ca^{2+}$ and $Mg^{2+}$ may be differentially compartmentalized (Grubbs et al., 1984), it is possible that under certain conditions $Mg^{2+}$ may compete effectively with $Ca^{2+}$ for sites I and II of CaM. We further evaluate this possibility in the next section.

*Could Sites I and II Be "$Mg^{2+}$–$Ca^{2+}$ Sites"?* An important question that has received broad attention is whether it is necessary to fill all four sites with $Ca^{2+}$ in order to activate calmodulin. A $Ca^{2+}$-independent calmodulin binding protein has been purified from bovine cerebral cortex membranes (Andreasen et al., 1983). However, detailed kinetic analysis have suggested that CaM·$Ca_3^{2+}$ or CaM·$Ca_4^{2+}$ is the activator in most calmodulin-dependent enzymes such as cAMP-dependent phosphodiesterase (Cox et al., 1983; Huang et al., 1981), phosphorylase *b* kinase (Burger et al., 1983), $Ca^{2+}$-dependent, $Mg^{2+}$-activated ATPase (Cox et al., 1982), and myosin light chain kinase (Blumenthal & Stull, 1982). The detailed mechanism of these enzymes have been reviewed recently (Manalan & Klee, 1984; Cox et al., 1984).

Since these enzymes also require $Mg^{2+}$, the kinetic assays were usually carried out in the presence of several millimolar $Mg^{2+}$. On the basis of our $^{25}$Mg NMR results, it may not be impossible that CaM·$Ca_2^{2+}$·$Mg_2^{2+}$, like CaM·$Ca_3^{2+}$ or CaM·$Ca_4^{2+}$, is an activator of the enzymes. Consider, for example, the standard assay condition for cAMP-dependent phosphodiesterase used in Chao et al. (1984). The assay mixture contained $9 \times 10^{-8}$ M CaM, 3 mM $Mg^{2+}$, 40 mM Tris-HCl (pH 8.0), 2 mM cyclic AMP, 0.12 μg of phosphodiesterase, and varying concentrations of $Ca^{2+}$. The activity of the phosphodiesterase reaches maximum and levels off at $[Ca^{2+}] \approx 10$ μM. If the relative affinity between $Ca^{2+}$ and $Mg^{2+}$ to CaM is unchanged when CaM is associated with the enzyme, then at least half of sites I and II should be occupied by $Mg^{2+}$ when $[Ca^{2+}] \approx 10$ μM, although further increase of $[Ca^{2+}]$ will eventually replace all $Mg^{2+}$ in these sites. In another example, calmodulin was found to activate bacterial adenylate cyclase in the absence of $Ca^{2+}$, although the concentration of CaM required for activation was ca. $10^3$ times higher than when $Ca^{2+}$ was present (Greenlee et al., 1982; Kilhoffer et al., 1983). The "calcium-free CaM" considered by the authors could indeed be CaM·$Mg_2^{2+}$ or CaM·$Mg_3^{2+}$, since their assay mixtures contained 10 mM $Mg^{2+}$.

The above argument may not hold if CaM discriminates $Ca^{2+}$ and $Mg^{2+}$ to a greater extent when it is complexed to the enzyme. However, another question may be raised: if only CaM·$Ca_3^{2+}$ and CaM·$Ca_4^{2+}$ bind to and activate the enzyme, then binding of the third and the fourth $Ca^{2+}$ ions to CaM will have to await the release of $Mg^{2+}$ from CaM. One of the many possible reasons for $Ca^{2+}$ to be a "second messenger" could be that substitution of inner sphere water occurs at a rate of ca. $10^{8.4}$ $s^{-1}$ for $Ca^{2+}$ (Frey & Stuehr, 1974), which makes the "on-rate" of $Ca^{2+}$ to calmodulin essentially diffusion controled (Forsén et al., 1983b). Since sites III and IV of CaM can be protected from high physiological concentration of $Mg^{2+}$, it would be hard to rationalize binding of $Mg^{2+}$ to sites I and II, with such a slow off-rate that binding of $Ca^{2+}$ can be severely hampered, without any purpose.

With such questions in mind, it seems relevant to consider skeletal muscle troponin C (TnC), which also possesses two strong $Ca^{2+}$ sites (sites III and IV, $K_a = 2.1 \times 10^7$ $M^{-1}$) and two lower affinity $Ca^{2+}$ sites (sites I and II, $K_a = 3.2 \times 10^5$ $M^{-1}$) (Potter & Gergely, 1975). Sites III and IV are usually called "$Ca^{2+}$–$Mg^{2+}$ sites", since they also bind $Mg^{2+}$, though with lower affinity ($K_a = 5 \times 10^3$ $M^{-1}$) (Potter & Gergely, 1975). On the basis of cation-exchange rates and the rates of conformational changes obtained from fluorescence stopped-flow analysis, Johnson et al. (1979) suggested that the $Ca^{2+}$-specific sites are the regulatory sites for skeletal muscle contraction and that the $Ca^{2+}$–$Mg^{2+}$ sites are probably always occupied by either $Ca^{2+}$ or $Mg^{2+}$ in vivo and serve to maintain the protein in a conformation ready for the regulatory event. On the basis of our $^{25}$Mg NMR results of CaM and considering the similarity between TnC and CaM in their properties in binding $Ca^{2+}$ and $Mg^{2+}$, we propose the following hypothesis: at least in some systems, sites I and II of CaM are always occupied by $Mg^{2+}$ in vivo and play a structural role (thus these sites should be called "$Mg^{2+}$–$Ca^{2+}$ sites") and sites III and IV are the regulatory sites. A possible structural role of sites I and II may be to maintain CaM in a conformation ready to interact with the target enzyme. It is not impossible that in certain systems CaM·$Mg_2^{2+}$ is already complexed to its target enzyme before the stimulus (such a binding could be weak and not detectable by gel filtration or electrophoresis), such that there is no need for CaM to "travel" to its target after stimulation. The rate of diffusion of calmodulin should be slower than the on-rate of $Ca^{2+}$ by a factor of ca. 10. When the $Ca^{2+}$ concentration is increased during stimulation, some or all of the $Mg^{2+}$ in sites I and II may be replaced by $Ca^{2+}$, but such a replacement may or may not have structural or regulatory effect, depending on the various systems.

Considering the diversity and complexity in the biological functions of calmodulin, we are not attempting to generalize its mechanism by the simple hypothesis. Rather, it is just a "working hypothesis" based on the data of this work and to be further tested in the future. The recent finding by Kincaid and Vaughan (1986) that less $Ca^{2+}$ is required for binding of calmodulin with calcineurin than for activation of calcineurin by calmodulin seems to lend some support to the above hypothesis. Whether it is a viable hypothesis or not, the binding of $Mg^{2+}$ to calmodulin should be further investigated, both biochemically and biophysically. For example, the conformation of CaM·$Ca_2^{2+}$·$Mg_2^{2+}$ should be compared with that of CaM·$Ca_4^{2+}$. In addition, the effect of monovalent cations such as $K^+$, which has been shown to accelerate the rate of the slow process and reduce the rate of the fast process in the

binding of $Ca^{2+}$ to CaM (Martin et al., 1985; Teleman et al., 1986), should also be evaluated for $Mg^{2+}$.

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