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

4 AUTHORS, INCLUDING:

Sture Forsén
Lund University
319 PUBLICATIONS **9,798** CITATIONS

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

Ming-Daw Tsai,* Torbjörn Drakenberg, Eva Thulin, and Sture Forsén

Physical Chemistry 2, Chemical Center, University of Lund, S-22100 Lund, Sweden
Received November 24, 1986; Revised Manuscript Received February 12, 1987

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. 5×10^5 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

---

[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; χ_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 $^{25}Mg^{2+}$; ω, Larmor velocity; τ_c, correlation time; Q, quadrupole moment; P_A and P_B, molar fraction of free and bound ions, respectively.

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 $[Mg^{2+}]/[protein] = 2.0$, 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* δ_A *and* δ_B. Values of 0 and 20 Hz, respectively, were used. The δ_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 χ_B is the quadrupolar coupling constant for the bound $^{25}Mg^{2+}$.

(2) The τ_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$_2$C (Teleman et al., 1986) (which is also assumed for TR$_1$C). In other words, it is assumed that both Mg^{2+} and Ca^{2+} are bound rigidly, such that the τ_c for the bound ion approximates the τ_c of the protein itself. If the bound Mg^{2+} possesses internal rotational freedom relative to the protein, χ_B will be underestimated, but k_{off} at 25 °C and binding constant K_a will not be affected. The temperature dependence of τ_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 ΔG values used in the analysis were calculated from the

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.

τ_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:

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

(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 $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ (which define k_{off}), χ_B, and K_a. In general, k_{off} is best defined by the "slow" region of the temperature curve, χ_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 χ_B. The $\Delta S^{\ddagger}$ may have larger errors, and the physical meaning for the strongly negative $\Delta S^{\ddagger}$ 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·Ca$_2^{2+}$. Figure 1

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	χ_B (MHz)^a	R_{2B} (kHz)	$\Delta\nu_B$ (kHz)
$CaM \cdot Ca_2^{2+b}$	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
$CaM \cdot La_2^{3+}$	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.

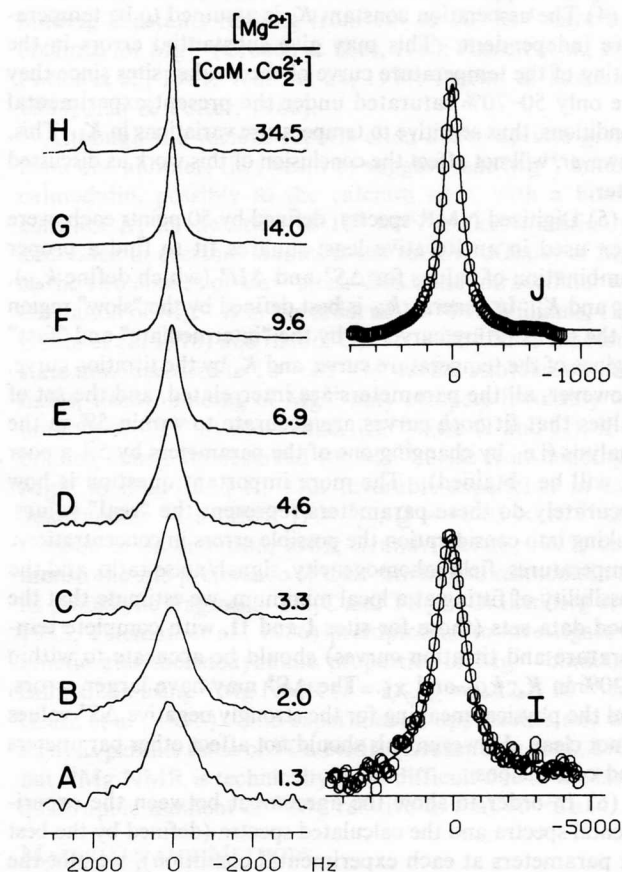FIGURE 1: ^{25}Mg NMR spectra (at 22.15 MHz, 25 °C) of Mg^{2+} at various ratios of $[Mg^{2+}]/[CaM \cdot Ca_2^{2+}]$ (A–H), which correspond to half of the experimental points (every other point) in Figure 2A. The detailed conditions are described under Materials and Methods. Spectra I and J are the 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.

shows some of the ^{25}Mg NMR spectra at different ratios of $[Mg^{2+}]/[CaM \cdot Ca_2^{2+}]$. The line widths ($\Delta\nu$) of the ^{25}Mg NMR signals are plotted as a function of the $[Mg^{2+}]/[CaM \cdot Ca_2^{2+}]$ ratios in Figure 2A. The temperature dependence of $\Delta\nu$ at $[Mg^{2+}]/[CaM \cdot Ca_2^{2+}] = 5.24$ 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+}
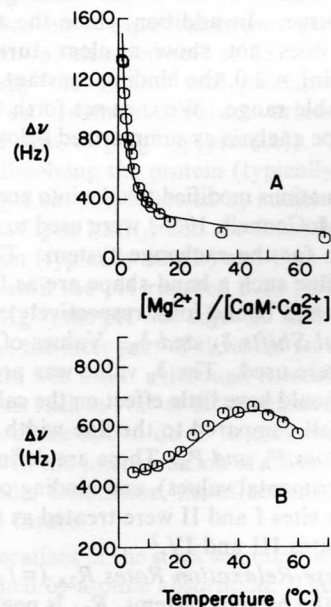

FIGURE 2: Titration curve (A) and temperature curve (B) of Mg^{2+} binding to $CaM \cdot Ca_2^{2+}$. The titration curve was obtained at 25 °C, with $[CaM \cdot Ca_2^{2+}]$ varying from 0.98 mM at the first point to 0.78 mM at the last point. The temperature curve was obtained at $[Mg^{2+}]/[CaM \cdot Ca_2^{2+}] = 5.24$. 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.

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
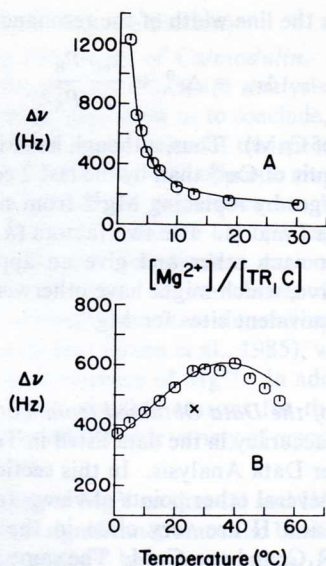
FIGURE 3: Titration curve (A) and temperature curve (B) of Mg^{2+} binding to TR$_1$C. The titration curve was obtained at 25 °C, with [TR$_1$C] varying from 0.54 mM at the first point to 0.51 mM at the last point. The temperature curve was obtained at [TR$_1$C] = 0.57 mM and [Mg^{2+}] = 2.32 mM. Circles 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 Δν shown include a line broadening of 100 Hz.

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$_1$C to further confirm the binding of Mg^{2+} to sites I and II of CaM. The titration curve and the temperature dependence in Δν are plotted in Figure 3 (A and B, respectively) and fitted with the total line-shape analysis. The relatively sharp decrease in Δν 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 Δν. 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·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 × 10^5 M^{-1} for the high-affinity sites (I, II) and the low-affinity sites (III, IV), respectively (Wang et al., 1984). Using ^{1}H 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·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·La$_2^{3+}$ is much weaker than the corresponding binding to CaM·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 Δν is smaller in this case, the
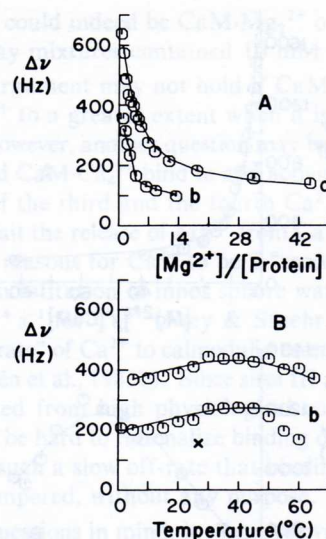

FIGURE 4: Titration curves (A) and temperature curves (B) of Mg^{2+} binding to CaM·La$_2^{3+}$ (curve a, the upper curve in both A and B) and to TR$_2$C (curve b, the lower curve in both A and B). The titration curves were obtained at 25 °C. Circles are experimental points, and solid curves are obtained by connecting the calculated points from the iterative fitting. For CaM·La$_2^{3+}$ (curves a), the starting and ending [CaM·La$_2^{3+}$] were 1.12 and 0.96 mM, respectively, the temperature curve was obtained at [Mg^{2+}]/[CaM·La$_2^{3+}$] = 2.85, and the Δν include a line broadening of 100 Hz. For TR$_2$C (curves b), the starting and ending [TR$_2$C] were 1.22 and 0.70 mM, respectively, the temperature curve was obtained at [Mg^{2+}]/[TR$_2$C] = 2.12, and the Δν include a line broadening of 50 Hz. The extra point (×) at 25 °C in temperature curve b for TR$_2$C 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$_2$C.

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$_2$C). The weak binding of Mg^{2+} to sites III and IV of CaM was also confirmed with the C-terminal tryptic fragment TR$_2$C, 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 Δν at higher temperatures was again found to be caused by partial denaturation of TR$_2$C (even though no precipitate formed), since the Δν 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 χ_B, we used the same χ_B as in TR$_1$C (1.5 MHz) and obtained values of k_{off} and K_a comparable to those of CaM·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 χ_B in both sites. On the basis of the larger binding constants in CaM·Ca$_2^{2+}$ and TR$_1$C, compared to those of CaM·La$_2^{3+}$ and TR$_2$C, 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 χ_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·La$_2^{3+}$ and TR$_2$C 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-
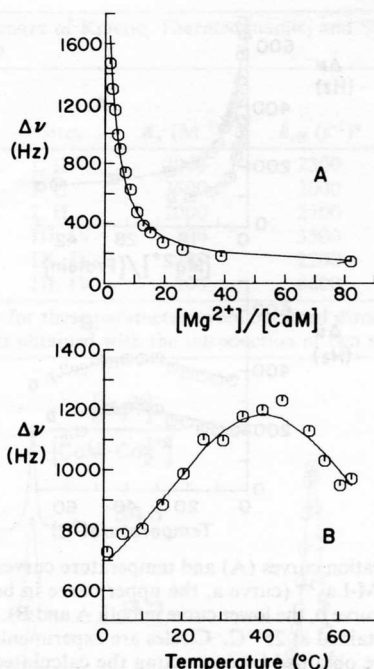
FIGURE 5: Titration curve (A) and temperature curve (B) of Mg^{2+} binding to apo-CaM. The titration curve was obtained at 25 °C, with [CaM] varying from 0.75 to 0.65 mM. The temperature curve was obtained at [CaM] = 1.0 mM and [Mg^{2+}] = 5.0 mM. Circles are experimental points, and solid curves are 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).
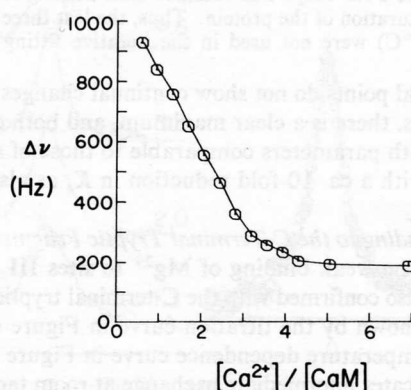

FIGURE 6: Addition of $CaCl_2$ to Mg^{2+}·CaM to show competition between Ca^{2+} and Mg^{2+}. The experiment was performed at 25 °C, pH 7.9, with [CaM] = 0.95 mM, [Mg^{2+}] = 5.0 mM, and varying ratios of [Ca^{2+}]/[CaM]. The $\Delta\nu$ include a line broadening of 100 Hz.

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+}

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

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+}.

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 χ_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 χ_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 χ_B of Mg^{2+} is greater than that of Ca^{2+} by ca. 50%. The difference between the χ 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 χ for small ions is governed by

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

If τ_c is assumed to be the same, $(\chi_{Mg}/\chi_{Ca})^2$ should be ca. 12. By such a rough estimation, χ_{Mg}/χ_{Ca} should be ca. 3.5, and the χ_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" χ_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) $\times 10^7$ s^{-1} M^{-1} for sites I and II and ca. (0.5–2.0) $\times 10^6$ 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 $\sim 10^5$ 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 $\times 10^7$ 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 $\times 10^3$ s^{-1} and 1.6 $\times 10^7$ 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 × 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 × 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+}] ≈ 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+}] ≈ 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 × 10^7 M^{-1}) and two lower affinity Ca^{2+} sites (sites I and II, K_a = 3.2 × 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 × 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+}.

REFERENCES

Andersson, A., Forsén, S., Thulin, E., & Vogel, H. J. (1983) Biochemistry 22, 2309-2313.

Andersson, T., Drakenberg, T., Forsén, S., Thulin, E., & Swärd, M. (1982a) J. Am. Chem. Soc. 104, 576-581.

Andersson, T., Drakenberg, T., Forsén, S., & Thulin, E. (1982b) Eur. J. Biochem. 126, 501-505.

Andreasen, T. J., Luetje, C. W., Heideman, W., & Storm, D. R. (1983) Biochemistry 22, 4615-4618.

Babu, Y. S., Sack, J. S., Greenhough, T. J., Bugg, C. E., Means, A. R., & Cook, W. J. (1985) Nature (London) 315, 37-40.

Blumenthal, D. K., & Stull, J. T. (1982) Biochemistry 21, 2386-2391.

Burger, D., Stein, E. A., & Cox, J. A. (1983) J. Biol. Chem. 258, 14733-14739.

Chao, S.-H., Suzuki, Y., Zysk, J. R., & Cheung, W. Y. (1984) Mol. Pharmacol. 26, 75-82.

Cox, J. A., Malone, A., & Stein, E. A. (1981) J. Biol. Chem. 256, 3218-3222.

Cox, J. A., Comte, M., & Stein, E. A. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 4265-4269.

Cox, J. A., Comte, M., Malone, A., Burger, D., & Stein, E. A. (1984) Met. Ions Biol. Syst. 17, 215-273.

Crouch, T. H., & Klee, C. B. (1980) Biochemistry 19, 3692-3698.

Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D., & Means, A. R. (1977) J. Biol. Chem. 252, 8415-8422.

Diebler, H., Eigen, M., Ilgenfritz, G., Maass, G., & Winkler, R. (1969) Pure Appl. Chem. 20, 93-115.

Drakenberg, T., Forsén, S., & Lilja, H. (1983) J. Magn. Reson. 53, 412-422.

Drakenberg, T., Andersson, T., Forsén, A., & Wieloch, T. (1984) Biochemistry 23, 2387-2392.

Eigen, M. (1963) Pure Appl. Chem. 6, 97-115.

Eigen, M., & Tamm, K. (1962) Z. Elektrochem. 66, 93-107.

Forsén, S., Andersson, A., Drakenberg, T., Teleman, O., Thulin, E., & Vogel, H. J. (1983a) in Calcium Binding Proteins (De Bernard, B., Scottocasa, G. L., Sandri, G., Carafoli, E., Taylor, A. N., Vanaman, T. C., & Williams, R. J. P., Eds.) pp 121-131, Elsevier, Amsterdam.

Forsén, S., Andersson, T., Drakenberg, T., Lilja, H., & Thulin, E. (1983b) Period. Biol. 85 (Suppl. 2), 31-42.

Forsén, S., Vogel, H. J., & Drakenberg, T. (1986) in Calcium and Cell Function (Cheung, W. Y., Ed.) Vol. 6, pp 113-157, Academic, New York.

Frey, C. M., & Stuehr, J. (1974) Met. Ions Biol. Syst. 1, 51.

Greenlee, D. V., Andreasen, T. J., & Storm, D. R. (1982) Biochemistry 21, 2759-2764.

Grubbs, R. D., Collins, S. D., & Maquire, M. E. (1984) J. Biol. Chem. 259, 12184-12192.

Grundvik, P., Gustavsson, M., Lindgren, I., Olsson, G., & Robertsson, L. (1979) Phys. Rev. Lett. 42, 1528-1531.

Gupta, R. K., Benovic, J. L., & Rose, Z. B. (1978) J. Biol. Chem. 253, 6172-6176.

Haiech, J., Klee, C. B., & Demaille, J. G. (1981) Biochemistry 20, 3890-3897.

Halle, B., & Wennerström, H. (1981) J. Magn. Reson. 44, 89-100.

Huang, C. Y., Chau, V., Chock, P. B., Wang, J. H., & Sharma, R. K. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 871-874.

Iida, S., & Potter, J. D. (1986) J. Biochem. (Tokyo) 99, 1765-1772.

Ikura, M., Hiraoki, T., Hikichi, K., Minowa, O., Yamaguchi, H., Yazawa, M., & Yagi, K. (1984) Biochemistry 23, 3124-3128.

Johnson, J. D., Charlton, S. C., & Potter, J. D. (1979) J. Biol. Chem. 254, 3497-3502.

Kilhoffer, M.-C., Demaille, J. G., & Gerard, D. (1980a) FEBS Lett. 116, 269-272.

Kilhoffer, M.-C., Gerard, D., & Demaille, J. G. (1980b) FEBS Lett. 120, 99-103.

Kilhoffer, M.-C., Cook, G. H., & Wolff, J. (1983) Eur. J. Biochem. 133, 11-15.

Kincaid, R. L., & Vaughan, M. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 1193-1197.

Lindman, B., Forsén, S., & Lilja, H. (1977) Chem. Scr. 11, 91-92.

Lutz, O., Schwenk, A., & Uhl, A. (1975) Z. Naturforsch., A: Phys., Phys. Chem., Kosmophys. 30A, 1122-1127.

Manalan, A. S., & Klee, C. B. (1984) Adv Cyclic Nucleotide Protein Phosphorylation Res. 18, 227-278.

Martin, S. R., Andersson Teleman, A., Bayley, P. M., Drakenberg, T., & Forsén, S. (1985) Eur. J. Biochem. 151, 543-550.

McConnell, H. M. (1958) J. Chem. Phys. 28, 430-438.

Mulqueen, P., Tingey, J. M., & Horrocks, W. DeW., Jr. (1985) Biochemistry 24, 6639-6645.

Newton, D. L., Oldewurtel, M. D., Krinks, M. H., Shiloach, J., & Klee, C. B. (1984) J. Biol. Chem. 259, 4419-4426.

Ogawa, Y., & Tanokura, M. (1984) J. Biochem. (Tokyo) 95, 19-28.

Olsson, G., & Salomonson, S. (1982) Z. Physik, A 307, 99-107.

Potter, J. D., & Gergely, J. (1975) J. Biol. Chem. 250, 4628-4633.

Potter, J. D., Robertson, S. P., & Johnson, J. D. (1981) Fed. Proc., Fed. Am. Soc. Exp. Biol. 40, 2653.

Potter, J. D., Strang-Brown, P., Walker, P. L., & Iida, S. (1983) Methods Enzymol. 102, 135-143.

Seamon, K. (1980) Biochemistry 19, 207-215.

Shimizu, T., & Hatano, M. (1985) Inorg. Chem. 24, 2003-2009.

Sutoo, D., Akiyama, K., Fujii, N., & Matsushita, K. (1986) Jpn. J. Pharmacol. 40, 169-173.

Teleman, A. (1986) Ph.D. Dissertation, University of Lund.

Teleman, A., Drakenberg, T., & Forsén, S. (1986) Biochim. Biophys. Acta 873, 204-213.

Thulin, E., Andersson, A., Drakenberg, T., Forsén, S., & Vogel, H. J. (1984) Biochemistry 23, 1862-1870.

Tsai, T.-C., Hart, J., Jiang, R.-T., Bruzik, K., & Tsai, M.-D. (1985) Biochemistry 24, 3180-3188.

Vogel, H. J., & Forsén, S. (1986) Biol. Magn. Reson. 7, 247-307.

Vogel, H. J., Lindahl, L., & Thulin, E. (1983) FEBS Lett. 157, 241-246.

Wallace, R. W., Tallant, E. A., Dockter, M. E., & Cheung, W. Y. (1982) J. Biol. Chem. 257, 1845-1854.

Wang, C.-L. A. (1985) Biochem. Biophys. Res. Commun. 130, 426-430.

Wang, C.-L. A., Aquaron, P. R., Leavis, P. C., & Gergely, J. (1982) Eur. J. Biochem. 124, 7-12.

Wang, C.-L. A., Leavis, P. C., & Gergely, J. (1984) *Biochemistry 23*, 6410–6415.

Williams, R. J. P. (1980) in *Calcium-Binding Proteins: Structure and Function* (Siegel, F. L., Carafoli, E., Kretsinger, R. H., MacLennan, D. H., & Wasserman, R. H., Eds.) pp 3–10, Elsevier, Amsterdam.

Wolff, D. J., Poirier, P. G., Brostrom, C. O., & Brostrom, M. A. (1977) *J. Biol. Chem. 252*, 4108–4117.

Xu, Y. H., Gietzen, K., & Galla, H. J. (1983) *Int. J. Biochem. 5*, 154–158.