parentage and having C_2 symmetry as possible structures for the $W(bmpd)_2(mpic)_2$ complex. It is clear, however, that these new mixed-ligand eight-coordinate tungsten(IV) complexes are rigid on the NMR time frame as were the previously studied $W(mpic)_n(dcq)_{4-n}$ complexes.[1]

Future Studies. As yet, no single-crystal X-ray diffraction studies have been performed on group 6 mixed-ligand eight-coordinate complexes containing two different bidentate ligands. If suitable crystals of the $W(bmpd)_2(mpic)_2$ complex can be obtained, such a study is planned. In addition, since the interconversion of the α and β forms of the $W(bmpd)_2(mpd)_2$ complex appears to be a relatively slow process, attempts will be made to isolate single crystals of the two forms and perform X-ray diffraction studies on these complexes as well.

Because the visible spectrum of the α and β forms of the $W(bmpd)_2(mpd)_2$ complex are virtually identical, we were unable to conduct spectrophotometric kinetic studies on their interconversion. Recent studies in our laboratory indicate that 5-*tert*-butyl-2-hydroxypyrimidine (Hbhpd) and 5-*tert*-butyl-2-selenopyrimidine (Hbspd) also can be used to form tetrakis eight-coordinate tungsten(IV) complexes.[22] The preparation and isolation of the α and β forms of $W(bhpd)_2(bmpd)_2$, $W(bmpd)_2(bspd)_2$, and/or $W(bhpd)_2(bspd)_2$ would provide series better suited for spectrophotometric kinetic studies. Kinetic studies on all three of these series of complexes would provide information on the influence of the donor atom on the rate of interconversion. At least one such study is planned.

Finally, we plan to continue our efforts to synthesize a stable tungsten(IV) eight-coordinate complex containing a bidentate ligand that forms a six-membered chelate ring. This would permit us to explore the number, stereochemistry, and rigid character of mixed-ligand eight-coordinate tungsten(IV) complexes containing a four- and a six-membered chelate ring and a five- and a six-membered ring, in addition to a mixed-ligand system containing two different bidentate ligands both of which formed six-membered chelate rings.

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Magnetic Resonance Studies of Trifluoperazine–Calmodulin Solutions: ^{43}Ca, ^{25}Mg, ^{67}Zn, and ^{39}K Nuclear Magnetic Resonance

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Interactions of calmodulin (CaM) with Ca^{2+}, Mg^{2+}, Zn^{2+}, K^+, and an antagonist were studied with metal NMR methods. Line widths of ^{43}Ca, ^{25}Mg, ^{67}Zn, and ^{39}K NMR of free Ca^{2+}, Mg^{2+}, Zn^{2+}, and K^+ were markedly increased by adding CaM. However, the ^{43}Ca NMR line width of the Ca^{2+}–CaM solution was markedly decreased by adding trifluoperazine (TFP), probably due to the reduction of the Ca^{2+}-exchange rate. The line width of the ^{25}Mg NMR of the Mg^{2+}–CaM solution was remarkably decreased by adding $CaCl_2$. Adding TFP to the Mg^{2+}–CaM solution also decreased the line width of the ^{25}Mg NMR of the solution. However, the decrease in line width observed for ^{25}Mg NMR of the Mg^{2+}–CaM solution by adding TFP was smaller than that observed for ^{43}Ca NMR of the Ca^{2+}–CaM solution. The increase in line width of the ^{67}Zn NMR of free Zn^{2+} by adding CaM in *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethenesulfonic acid (HEPES)–Na^+ solution was larger than that in HEPES–K^+ solution. The line width shown by the Zn^{2+}–CaM solution containing HEPES–Na^+ was not changed by adding excess Ca^{2+}, while that in HEPES–K^+ solution was markedly decreased by adding excess Ca^{2+}. The line width of the ^{39}K NMR of the K^+–CaM solution was decreased by adding Ca^{2+}. From these and other spectral findings, the following suggestions were given: (1) The environment of the Ca^{2+} low-affinity site in CaM is markedly changed by TFP. (2) Mg^{2+} binds exactly to the Ca^{2+} binding site in CaM. (3) Mg^{2+} does not cause a specific conformational change of CaM, which is necessary for the specific TFP–CaM interaction. (4) K^+ binds to the Zn^{2+} binding site in CaM. (5) Zn^{2+} binds to the Ca^{2+} binding site of CaM in the HEPES–K^+ soluion, while this is not true in the HEPES–Na^+ solution. Therefore, the high utility of diamagnetic metal NMR has been demonstrated.

Introduction

Calmodulin (CaM)[1] is a ubiquitous and multifunctional regulatory protein and plays the central role in th regulation of cellular functions.[2-4] Ca^{2+} is essential for CaM–dependent functions. CaM has four Ca^{2+} binding sites, probably two of which are Ca^{2+} high-affinity sites and the others are Ca^{2+} low-affinity sites.[2-4] The structure of CaM is changed by Ca^{2+}, but a detailed conformational change of CaM caused by Ca^{2+} has not been shown yet. Bivalent metal cations other than Ca^{2+} such as Mg^{2+}, Mn^{2+}, or Zn^{2+} are known to activate the CaM functions or to bind to CaM.[5-10] It has been controversial whether Mg^{2+} binds exactly to the Ca^{2+} binding sites of CaM or not.[5-10] It was also suggested from CD and absorption spectroscopies that alkanine monovalent metal cations such as K^+, Na^+, etc., markedly change the conformation of CaM.[5-8] Thus, it will be necessary to study the exact binding sites of these monovalent and bivalent metal cations on CaM to understand their roles in physiological functions.

An antipsychotic drug, trifluoperazine (TFP), is a potent antagonist of CaM functions.[11,12] TFP will tightly bind to CaM,

(1) Abbreviations: CaM, calmodulin; TFP, trifluoperazine; K_d, dissociation constant; CD, circular dichroism; UV, ultraviolet; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethenesulfonic acid.

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probably through the hydrophobic interaction in the presence of Ca^{2+} as CaM-dependent enzymes do. The study of the interactions of CaM with TFP will provide a good research model for elucidating the structure–function relationship of CaM in cell functions. From ^{19}F NMR studies, it was suggested that KCl markedly affects the interaction of TFP with CaM.[13] In our preceding paper,[14] we demonstrated in terms of induced CD spectroscopy that Mn^{2+}, Cd^{2+}, and Zn^{2+} enhance the TFP–CaM interaction as Ca^{2+} does and that the enhancement of the TFP–CaM interaction caused by these bivalent cations is fairly influenced by the ionic strength of the solution. It was also suggested from induced CD spectral studies[14] that Mg^{2+} does not so much enhance the TFP–CaM interaction as Ca^{2+} does, irrespective of the ionic strength of the solution. Mutual relationships of bivalent cations such as Ca^{2+}, Zn^{2+}, and Mg^{2+} and the monovalent cation K^+ in the TFP–CaM interaction seemed to be quite interesting for elucidating the role of Ca^{2+} in intracellular functions.

Ca^{2+}, Mg^{2+}, Zn^{2+}, and K^+ are diamagnetic and nonchromophoric. Quadrupolar metal NMR is a highly useful direct method for studying the dynamic and static behavior of diamagnetic quadrupolar metal cations interacting with protein and enzymes.[15] We have already utilized ^{43}Ca and ^{25}Mg NMR spectroscopies for studying the environment of Ca^{2+} in CaM or other protein solutions.[9,16–19] It was also found that ^{67}Zn NMR spectroscopy can be successfully applied to Zn^{2+}–enzyme or Zn^{2+}–protein systems for studying the environment or behavior of Zn^{2+} interacting with the enzyme or protein.[17,18,20–22]

Following our successive ^{43}Ca, ^{25}Mg, and ^{67}Zn NMR studies, we present here ^{43}Ca, ^{25}Mg, ^{67}Zn, and ^{39}K NMR studies of Ca^{2+}–, Mg^{2+}–, Zn^{2+}–, and K^+–CaM solutions in the presence or absence of TFP. This study has shown that Mg^{2+} binds exactly to the Ca^{2+} binding site(s) of CaM and that TFP binds to the Mg^{2+}–CaM complex. Zn^{2+} and K^+ also seemed likely to bind to the Ca^{2+} binding site(s) of CaM under our experimental conditions. It was also found that the environment of the metal binding site(s) of CaM is changed by TFP. We will discuss the mutual structure–function relationships of those bivalent or monovalent metal cations and CaM. The high utility of bivalent metal NMR for observing the environments of metal binding sites of proteins was shown.

Experimental Section

CaM was prepared from porcine brain by a modification of the methods as previously described.[23,24] Briefly, our method included precipitation with trichloroacetic acid and chromatography on phenyl Sepharose. The purity of the protein was checked by SDS gel electrophoresis. Activity of CaM was satisfactory before and after NMR measurements in terms of phosphodiesterate activity.[14]

^{43}Ca was purchased in 49.1% $CaCO_3$ purity from the Commissariat a L'Energie Atomique of France. ^{25}Mg was purchased in 95.66% MgO purity from Prochem. ^{67}Zn was purchased in 78.63% element (metal) from Prochem. The isotope-enriched $CaCO_3$, MgO, and Zn were dissolved in metal-free 1 M HCl and were neutralized to a final pH of 7.0 or 6.7 by adding 1 M NaOH. Doubly distilled water was used throughout the NMR experiments. Chelex-100 (Bio-Gel) was used to eliminate Ca^{2+} in solvent or protein.

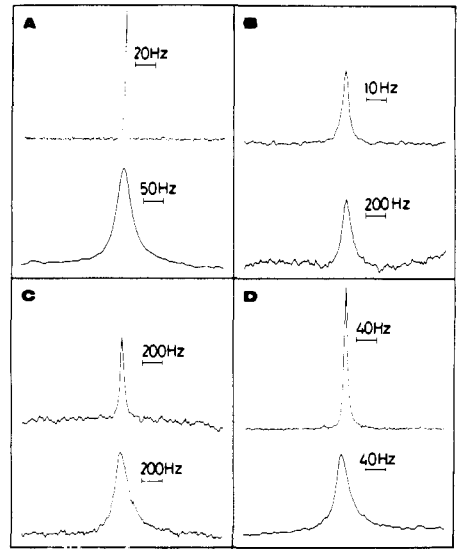

Figure 1. Typical ^{43}Ca, ^{25}Mg, ^{67}Zn, and ^{39}K NMR spectra of CaM solutions: (A) ^{43}Ca NMR spectra of enriched $^{43}Ca^{2+}$ (2.5 mM) [pH 7.0 (upper)] and enriched $^{43}Ca^{2+}$ (2.5 mM)–CaM (0.2 mM) [pH 7.0 (lower)], number of scans 10^4 (upper) and 4×10^5 (lower); (B) ^{25}Mg NMR spectra of enriched $^{25}Mg^{2+}$ (3 mM) [pH 7.0 (upper)] and enriched $^{25}Mg^{2+}$ (3 mM)–CaM (0.1 mM) [pH 7.0], number of scans 10^4 (upper) and 4×10^5 (lower); (C) ^{67}Zn NMR spectra of enriched $^{67}Zn^{2+}$ (4.5 mM) (upper) and enriched $^{67}Zn^{2+}$ (4.5 mM)–CaM (3 μM) (lower) in 50 mM HEPES–Na^+ (pH 6.7) buffer, number of scans 4×10^4 (upper) and 2×10^5 (lower); (D) ^{39}K NMR spectra of KCl (10 mM) [pH 7.0 (upper)] and K^+ (10 mM)–CaM (0.25 mM) [pH 7.0 (lower)], number of scans 10^4 (upper) and 2×10^5 (lower). Other spectral conditions: temperature, 294 K; 90° pulse, 70–80 μs; acquisition time, 0.12–4 s; size, 16*k*; sweep width, 2000–5000 Hz; exponential line broadening, 0.4–10 Hz.

^{43}Ca, ^{25}Mg, ^{67}Zn, and ^{39}K NMR spectra were accumulated on a Bruker CXP-300 FT NMR spectrometer at 20.19, 18.36, 18.77, and 14.00 MHz, respectively, in spinning 10-mm sample tubes with external D_2O for a frequency lock at 294 K. A transmitter provided 90° pulse widths of nearly 80 μs for ^{43}Ca, ^{25}Mg, and ^{67}Zn nuclei and of nearly 70 μs for ^{39}K nuclei at a peak-to-peak voltage of 300 V. Typical spectra consisted of more than 10^4 transients for ^{43}Ca, ^{25}Mg, and ^{39}K NMR and more than 10^5 transients for ^{67}Zn NMR before obtaining a signal/noise of more than 8, using 1*K*–16*K* data points over a 2000–5000 Hz spectral window in quadrature detection mode. The signal/noise ratio was improved by exponential multiplication that introduced 0.4–10-Hz line broadenings. The repetition time was changed from 0.12 to 4 s, depending on the line widths of spectra of sample solutions to fit sufficient delay time. For ^{67}Zn and ^{39}K NMR spectroscopies, a dead time of more than 400 μs was necessary due to accumulated ring down in the probe after more than 10^5 transients. For ^{43}Ca and ^{25}Mg NMR spectroscopies, a dead time of 50–200 μs was necessary to obtain satisfactory spectra. NMR spectra were obtained just after sample preparations and were obtained 1 and 12 h after sample preparations. No time-dependent change of NMR spectra was observed. Activities of CaM were not changed for 12 after sample preparations.[14] The pH values of sample solutions for ^{43}Ca and ^{25}Mg NMR spectra were strictly adjusted to pH 7.0 ± 0.1 before NMR measurements by 0.01 M NaOH or 0.01 M HCl. For ^{67}Zn NMR spectral measurements, we always used 50 mM HEPES–Na^+ or HEPES–K^+ buffer adjusted to pH 6.7 ± 0.1 because $^{67}Zn^{2+}$ in distilled water, pH >6.5, gave quite broad ^{67}Zn NMR spectra that were very hard to observe. For ^{39}K NMR spectral measurements, 0.01 M NaOH or 0.01 M HCl was sometimes used to adjust the pH value to pH 7.0 ± 0.1, which may change the ^{39}K NMR spectra of sample solutions as will be mentioned in later text. We strictly checked the resolution of the NMR spectra by using the lock signal height of external D_2O for each NMR sample before measuring NMR spectra. Thus, experimental data were very reproducible.

Results

^{43}Ca NMR. It is known that KCl markedly affects the conformation of CaM and the binding behavior of bivalent metal cations to CaM.[5–8] Interaction of TFP with CaM is also markedly affected by KCl in terms of ^{19}F NMR spectra[13,24] and induced CD spectra.[14] We have studied in this paper the effect of KCl on ^{43}Ca NMR spectra of the Ca^{2+}–CaM solution. Figure 1A

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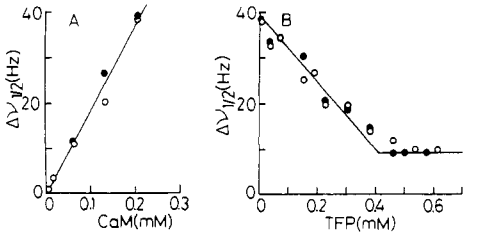

Figure 2. ^{43}Ca NMR spectra of Ca^{2+}–CaM solutions: (A) changes in line width of the ^{43}Ca NMR for enriched $^{43}Ca^{2+}$ (2.5 mM) by adding CaM in the absence (○) and presence (●) of 0.2 M KCl, pH 7.0; (B) changes in line width of the ^{43}Ca NMR for enriched $^{43}Ca^{2+}$ (2.5 mM)–CaM (0.20 mM) by adding TFP in the absence (○) and presence (●) of 0.2 M KCl, pH 7.0.

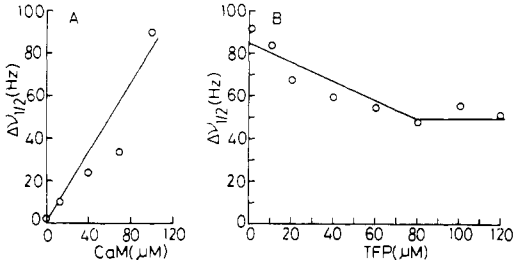

Figure 3. ^{25}Mg NMR spectra of Mg^{2+}–CaM solutions: (A) changes in line width of the ^{25}Mg NMR for enriched $^{25}Mg^{2+}$ (3 mM) by adding CaM in distilled water, pH 7.0; (B) changes in line width of the ^{25}Mg NMR for enriched $^{25}Mg^{2+}$ (3 mM)–CaM (0.1 mM) by adding TFP in distilled water, pH 7.0.

shows representative ^{43}Ca NMR spectra of free Ca^{2+} and Ca^{2+}–CaM solutions. Figure 2A shows the change in line width of ^{43}Ca NMR signal of free Ca^{2+} caused by adding CaM. The line width of free Ca^{2+} was markedly increased by adding CaM. The increase of the line width caused by adding CaM was somewhat smaller than that by *Tetrahymena* CaM.[9] We measured the line widths at four different temperatures. By decreasing the temperature from 294 to 276 K of the Ca^{2+} (2.5 mM)–CaM (0.20 mM) solution, the line width of the solution was decreased by 20 Hz. Experimental points of the line widths vs. reciprocal of temperature formed a straight line (cf. Figure A; supplementary material). Apparent increase of the ^{43}Ca NMR line width caused by adding CaM in distilled water was the same as that in 0.2 M KCl solution at pH 7.0 (Figure 2A). To adjust pH of distilled water and the 0.2 M KCl solution to 7.0, drops of diluted NaOH solution (<0.1 mM) were added to the solutions. The addition of diluted NaOH did not influence the titration behavior of ^{43}Ca NMR spectra. Figure 2B shows the line width change of the ^{43}Ca NMR of the Ca^{2+}–CaM solution caused by adding TFP. The line width of the spectrum of the Ca^{2+}–CaM solution was markedly decreased by adding TFP. The decrease of the line width was saturated at [TFP]/[CaM] molar ratio of approximately 2. The spectral change caused by adding TFP in distilled water was the same as that in 0.2 M KCl solution. Thus, it seemed likely that KCl does not influence the binding behavior of Ca^{2+} to CaM and the environmental change of Ca^{2+} in CaM caused by adding TFP in terms of the ^{43}Ca NMR.

^{25}Mg NMR. Figure 1B shows typical ^{25}Mg NMR spectra of free Mg^{2+} and Mg^{2+}–CaM solutions. Figure 3A shows the line width change of the ^{25}Mg NMR of free Mg^{2+} caused by adding CaM. The line width of the spectrum of Mg^{2+} was markedly increased by adding CaM. The extent of the line width increase of the ^{25}Mg NMR of free Mg^{2+} caused by adding CaM was three times as large as that of the ^{43}Ca NMR of free Ca^{2+} caused by adding CaM. We measured the line widths at four different temperatures. By decreasing the temperature from 294 to 276 K of the Mg^{2+} (3 mM)–CaM (0.1 mM) solution, the line width of the solution decreased by 30 Hz. Experimental points of the line widths vs. reciprocal of temperature formed a straight line (cf. Figure A; supplementary material). By adding TFP to the Mg^{2+}–CaM solution, the line width of the ^{25}Mg NMR was decreased as shown in Figure 3B. The line width decrease of the ^{25}Mg NMR by adding TFP to the Mg^{2+}–CaM solution was not so marked as that of the ^{43}Ca NMR by adding TFP to the Ca^{2+}–CaM solution. The decrease of line width was saturated at [TFP]/[CaM] molar ratio of approximately unity.

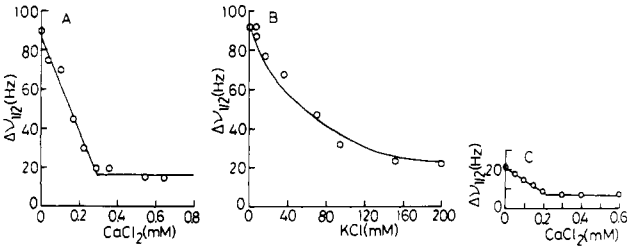

Figure 4. ^{25}Mg NMR spectra of Mg^{2+}–CaM solutions: (A) changes in line width of the ^{25}Mg NMR for enriched $^{25}Mg^{2+}$ (3 mM)–CaM (0.1 mM) by adding $CaCl_2$ in distilled water, pH 7.0; (B) changes in line width of the ^{25}Mg NMR for enriched $^{25}Mg^{2+}$ (3 mM)–CaM (0.1 mM) by adding KCl in distilled water, pH 7.0 [theoretical curve fit to the data on the assumption of simple binding, $\Delta\nu_{1/2} = \{\Delta\nu_{1/2}^{max}(1/K_d)[L]\}/\{1 + (1/K_d)[L]\}$, with K_d of 50 mM for KCl from CaM]; (C) changes in line width of the ^{25}Mg NMR for enriched $^{25}Mg^{2+}$ (3 mM)–CaM (0.1 mM)–KCl (0.2 M) by adding $CaCl_2$.

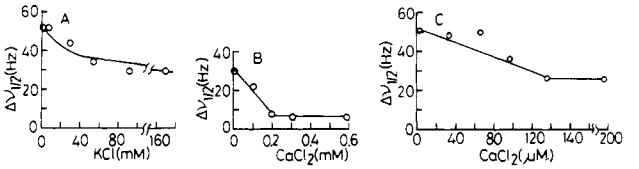

Figure 5. ^{25}Mg NMR spectra of Mg^{2+}–CaM–TFP solutions: (A) changes in line-width of the ^{25}Mg NMR for enriched $^{25}Mg^{2+}$ (3 mM)–CaM (0.1 mM)–TFP (0.12 mM) by adding KCl [theoretical curve fit to the data on the assumption of simple binding with K_d of 30 mM for KCl from CaM as in Figure 4B]; (B) changes in line width of the ^{25}Mg NMR for enriched $^{25}Mg^{2+}$ (3 mM)–CaM (0.1 mM)–TFP (0.12 mM)–KCl (170 mM) by adding $CaCl_2$; (C) changes in line width of the ^{25}Mg NMR for enriched $^{25}Mg^{2+}$ (3 mM)–CaM (0.1 mM)–TFP (0.12 mM) by adding $CaCl_2$.

To know whether or not Mg^{2+} is bound to the Ca^{2+} binding site(s) of CaM, ^{25}Mg NMR spectral titration of the Mg^{2+}–CaM solution by adding Ca^{2+} was studied. The line width of the ^{25}Mg NMR of the Mg^{2+}–CaM solution was markedly decreased by adding Ca^{2+} as shown in Figure 4A. The decrease in line width of the Mg^{2+} (3 mM)–CaM (0.1 mM) solution was saturated at 0.3 mM Ca^{2+}. We also studied the effect of KCl on the ^{25}Mg NMR of the Mg^{2+}–CaM solution. Addition of KCl to the Mg^{2+}–CaM solution markedly decreased the line width of the ^{25}Mg NMR of the Mg^{2+}–CaM solution (Figure 4B). Since the concentration of free K^+ is very large compared with that of bound K^+, the ^{25}Mg NMR titration curve caused by adding KCl (Figure 4B) is fitted to the hyperbolic curve. By asssuming simple binding equation, $\Delta\nu_{1/2} = \{\Delta\nu_{1/2}^{max}(1/K_d)[L]\}/\{1 + (1/K_d)[L]\}$, where $\Delta\nu_{1/2}$ is the line width, $\Delta\nu_{1/2}^{max}$ is the maximum change of the line width, and [L] is the concentration of KCl, K_d was estimated to be approximately 50 mM. Note that titration points form a straight line when the binding capacity of metal cations is very high ($K_d < 10^{-5}$ M) as observed in Figures 2B and 4A. Addition of Ca^{2+} to the Mg^{2+}–CaM–KCl solution further decreased the line width of ^{25}Mg NMR of the solution (Figure 4C). The ^{25}Mg NMR spectral change of the Mg^{2+} (3 mM)–CaM (0.1 mM)–KCl (0.2 M) solution caused by adding Ca^{2+} occurred at 0.2 mM of Ca^{2+}.

We have studied the effects of KCl and Ca^{2+} on the ^{25}Mg NMR of the Mg^{2+}–CaM–TFP solution. Figure 5A shows that the line width of the ^{25}Mg NMR of the Mg^{2+}–CaM–TFP solution was decreased by adding KCl. A K_d value for KCl from Mg^{2+}–CaM–TFP was estiamted to be 30 mM, which is comparable to the K_d, 10 mM, of KCl from the TFP–CaM solution determined with induced CD spectra.[14] The decrease of the line width of the Mg^{2+}–CaM–TFP solution by adding KCl was much smaller than that observed for the Mg^{2+}–CaM solution by adding KCl (Figure 4B). Addition of Ca^{2+} to the Mg^{2+}–CaM–TFP–KCl solution

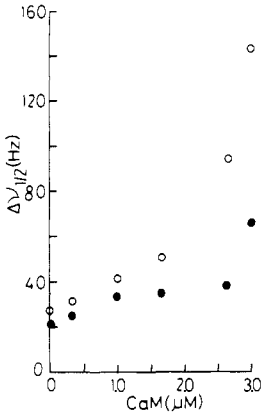

Figure 6. ^{67}Zn NMR spectra of Zn^{2+}–CaM solutions: changes in line width of the ^{67}Zn NMR for enriched $^{67}Zn^{2+}$ (4.5 mM) by adding CaM in 50 mM HEPES–Na^+ (pH 6.7) (○) and in 50 mM HEPES–K^+ (pH 6.7) (●).

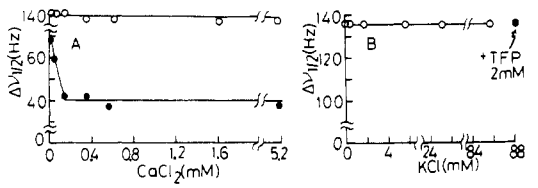

Figure 7. ^{67}Zn NMR spectra of Zn^{2+}–CaM solutions: (A) line width of the ^{67}Zn NMR for enriched $^{67}Zn^{2+}$ (4.5 mM)–CaM (3 μM) in the presence of $CaCl_2$ in 50 mM HEPES–Na^+ (pH 6.7) (○) and in 50 mM HEPES–K^+ (pH 6.7) (●); (B) line width of the ^{67}Zn NMR for enriched $^{67}Zn^{2+}$ (4.5 mM)–CaM (3 μM) in 50 mM HEPES–Na^+ (pH 6.7) in the presence of KCl (86 mM) (○) and in the presence of both KCl (86 mM) and TFP (2 mM) (●).

further decreased the line width of the ^{25}Mg NMR (Figure 5B), which was the same as that observed for the Mg^{2+}–CaM–KCl solution by adding Ca^{2+} (Figure 4C). The ^{25}Mg NMR spectral change of the Mg^{2+} (3 mM)–CaM (0.1 mM)–TFP (0.12 mM)–KCl (170 mM) solution caused by adding Ca^{2+} occurred at 0.2 mM Ca^{2+}. The line width change of the ^{25}Mg NMR of the Mg^{2+}–CaM–TFP solution caused by adding Ca^{2+} (Figure 5C) was less marked than that observed for the Mg^{2+}–CaM solution by adding Ca^{2+} (Figure 4A). The ^{25}Mg NMR spectral change of the Mg^{2+} (3 mM)–CaM (0.1 mM)–TFP (0.12 mM) solution occurred at approximately 0.12 mM Ca^{2+}.

^{67}Zn NMR. It has been reported that Zn^{2+} binds to the Ca^{2+} binding site(s) of CaM under certain conditions.[10,14] We have used ^{67}Zn NMR spectroscopy to study the interaction of Zn^{2+} with CaM or with CaM–TFP (Figure 1C). Since the ^{67}Zn NMR band of free Zn^{2+} in distilled water (pH >6.5) is very broad, probably due to its hydroxide form, we had to use a buffer solution for obtaining the ^{67}Zn NMR band of the Zn^{2+}–CaM solution with satisfactory signal/noise ratio. HEPES buffer seemed to be the most suitable buffer for studying ^{67}Zn NMR in aqueous solutions as already described.[17,21] It was first found through the work in this paper that ^{67}Zn NMR spectral behavior of the Zn^{2+}–CaM solution in HEPES–Na^+ buffer is quite different from that in HEPES–K^+ buffer. Namely, the line width increase of the ^{67}Zn NMR of free Zn^{2+} caused by adding CaM in HEPES–Na^+ buffer was larger than that in HEPES–K^+ buffer as seen in Figure 6. We measured spectra at four different temperatures. The ^{67}Zn NMR line width of the Zn^{2+} (4.5 mM)–CaM (3.0 μM) solution in 50 mM HEPES–K^+ or HEPES–Na^+ vs. the reciprocal of temperature formed a straight line and was decreased by 20 Hz by lowering the temperature from 294 to 274 K (cf. Figure A; supplementary material). Addition of Ca^{2+} to the Zn^{2+}–CaM–HEPES–Na^+ solution scarcely changed the line width of the ^{67}Zn NMR, while addition of Ca^{2+} to the Zn^{2+}–CaM–HEPES–K^+ solution markedly decreased the line width of the ^{67}Zn NMR of the solution (Figure 7A). Adding a large excess of KCl or TFP to the Zn^{2+}–CaM–Ca^{2+}–HEPES–Na^+ solution hardly changed

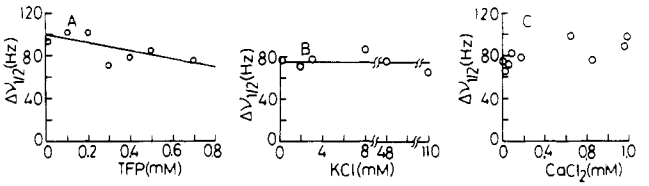

Figure 8. ^{67}Zn NMR spectra of Zn^{2+}–CaM–TFP solutions: (A) changes in line width of the ^{67}Zn NMR for enriched $^{67}Zn^{2+}$ (4.5 mM)–CaM (2.6 μM) in 50 mM HEPES–Na^+ (pH 6.7) by adding TFP; (B) line width of the ^{67}Zn NMR for enriched $^{67}Zn^{2+}$ (4.5 mM)–CaM (2.6 μM)–TFP (0.8 mM) in 50 mM HEPES–Na^+ (pH 6.7) in the presence of KCl; (C) line width of the ^{67}Zn NMR for enriched $^{67}Zn^{2+}$ (4.5 mM)–CaM (2.6 μM)–TFP (0.8 mM)–KCl (110 mM) in 50 mM HEPES–Na^+ (pH 6.7) in the presence of $CaCl_2$.

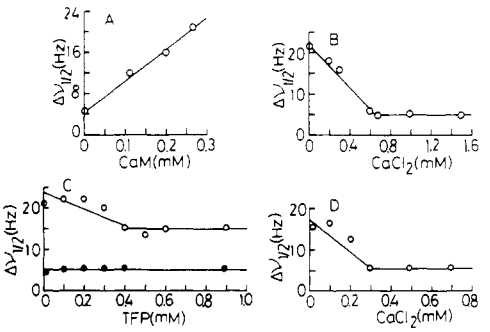

Figure 9. ^{39}K NMR spectra of K^+–CaM solutions: (A) changes in line width of the ^{39}K NMR for naturally abundant K^+ (10 mM) by adding CaM, pH 7.0; (B) changes in line width of the ^{39}K NMR for K^+ (10 mM)–CaM (0.26 mM) by adding $CaCl_2$, pH 7.0; (C) changes in line width of the ^{39}K NMR for K^+ (10 mM)–CaM (0.26 mM) (○) and for K^+ (10 mM)–CaM (0.26 mM)–$CaCl_2$ (1.5 mM) (●) by adding TFP, pH 7.0; (D) changes in line width of the ^{39}K NMR for K^+ (10 mM)–CaM (0.26 mM)–TFP (0.9 mM) by adding $CaCl_2$, pH 7.0.

the ^{67}Zn NMR spectra of the solution (Figure 7B). Figure 8A shows the effect of TFP on the ^{67}Zn NMR of the Zn^{2+}–CaM–HEPES–Na^+ solution. The line width of the ^{67}Zn NMR of this solution was decreased to a little extent by adding 0.25 mM TFP. Addition of KCl to the Zn^{2+}–CaM–TFP–HEPES–Na^+ solution scarcely decreased the line width of ^{67}Zn NMR of the solution. Addition of Ca^{2+} to the Zn^{2+}–CaM–TFP–HEPES–Na^+ solution did not result in a distinct change of the line width of the ^{67}Zn NMR of the solution as shown in Figure 8C.

^{39}K NMR. Since KCl markedly influences the interaction of TFP or bivalent metal cations with CaM,[5,7,8,13,25] we have obtained ^{39}K NMR spectra of various K^+–CaM–Ca^{2+} and K^+–CaM–TFP solutions (Figure 1D). The line width of the ^{39}K NMR of K^+ was increased by adding CaM (Figure 9A). However, the ^{39}K NMR change of free K^+ caused by adding CaM was much smaller that those observed for ^{43}Ca, ^{25}Mg, and ^{67}Zn NMR spectra of free metal cations caused by adding CaM. The ^{39}K NMR line width of the K^+–CaM solution was scarcely changed by lowering the temperature from 294 to 274 K. The broad line width of the ^{39}K NMR of the K^+–CaM solution was decreased by adding Ca^{2+} (Figure 9B). The ^{39}K NMR spectral change of the K^+ (10 mM)–CaM (0.26 mM) solution occurred at approximately 0.6 mM Ca^{2+}. Adding excess TFP to the K^+–CaM–Ca^{2+} solution did not change the ^{39}K NMR spectra (Figure 9C), while adding excess TFP to the K^+ (10 mM)–CaM (0.26 mM) solution decreased the line width of the ^{39}K NMR to a certain extent, with a saturation point of nearly 0.4–0.5 mM TFP. Adding Ca^{2+} to the K^+ (10 mM)–CaM (0.26 mM)–TFP (0.9 mM) solution decreased the line width of the ^{39}K NMR, with a saturation point of nearly 0.3 mM Ca^{2+} (Figure 9D).

Discussion

The line widths of the quadrupole metal cation–ligand complexes, which are associated with the transverse relaxation rate

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(T_2), are attributed to various causes such as (1) chemical exchange rate of the quadrupolar cation, (2) reorientational motion of the quadrupole nucleus, (3) reorientational motion of the whole metal–ligand complex, and (4) symmetry around the nucleus, which may affect the quadrupole coupling constant. Furthermore, the line widths can be affected by the fraction of bound cations in connection with equilibrium between free metal cation and metal cation bound to ligand.

The asymmetrical contribution to the quadrupolar relaxation of exchangeable metal ion is usually negligible.[15] Furthermore, since the concentration of the metal is in high excess of that of CaM under our experimental conditions, changes of the fraction of free or bound metal ions by changing the temperature hardly contribute to the line width of the metal–macromolecule solutions compared with the change of the exchange rate under the temperature we studied. By lowering the temperature of the solution, the line widths of the metal NMR (^{43}Ca, ^{25}Mg, ^{67}Zn) of metal–CaM solutions were decreased. This finding is in contrast with those observed for metal–small molecule (ATP or imidazole) solutions where the line widths are increased by lowering the temperature.[16,22] The line widths of the metal–small molecule solutions are dominated by a correlation time that describes the reorientation of the entire molecule.[15,16,22] On the other hand, the line widths of the metal–CaM solutions are dominated by the chemical exchange under the temperature we studied. The line broadenings of the ^{43}Ca, ^{25}Mg, and ^{67}Zn NMR in this study will be controlled by moderately fast exchange rates.

Note that, depending on the K_d value, the titration curves are sometimes fitted to the hyperbolic equation appearing in Figure 4B or by straight lines appearings, for example, in Figures 2B and 4A (cf. the ^{25}Mg NMR section in Results).

^{43}Ca NMR. From ^{43}Ca NMR studies, it was found that KCl does not influence the interaction of Ca^{2+} or TFP with CaM (Figure 2A,B). Ca^{2+}, which is bound to its high-affinity site ($K_d \simeq 10^{-7}$ M),[5] will not cause the ^{43}Ca NMR line broadening since the exchange rate of the Ca^{2+} ion is extremely slow, less than 50 s^{-1} at 30 °C or lower.[10,18] Thus, the broad-line ^{43}Ca NMR of the Ca^{2+}–CaM solution (Figure 1A) may reflect the binding of Ca^{2+} to the low-affinity site ($K_d \simeq 10^{-6}$ M for Ca^{2+})[5] of CaM. The environment of Ca^{2+} bound at its lower affinity site in CaM may not be influenced by excess KCl.

From equilibrium dialysis studies, it was suggested[5] that K_d of Ca^{2+} from Ca^{2+} high-affinity site(s) in CaM is 2×10^{-7} M and that from the Ca^{2+} low-affinity site is 1×10^{-6} M in the absence of appropriate amount of salts. In the presence of 0.1 M KCl, however, K_d of Ca^{2+} from CaM is 2×10^{-6} M and thus the high affinity ($K_d \simeq 10^{-7}$ M) of Ca^{2+} to CaM was not observed.[6] Thus, it seems very unlikely from equilibrium dialysis studies that only the environments of the Ca^{2+} high-affinity sites were influenced by KCl. ^{43}Ca NMR results described here are in accordance with those obtained with the equilibrium dialysis method.[5,6] From optical titration studies, Burger et al.[26] gave similar results. Thus, it was suggested here again that low ionic strength in aqueous protein solution may lead to different results on metal or drug binding to CaM, a highly charged protein, due to a Donnan-type effect.[26,27]

From the temperature-dependence study, it is suggested that the line width of the spectrum of the Ca^{2+}–CaM solution is controlled by the chemical exchange of Ca^{2+} from CaM. In the fast-exchange limit, the relaxation equation becomes $\Delta\nu_{1/2} = P_f\Delta\nu_{1/2f} + P_b\Delta\nu_{1/2b}$, where P_f is the fraction of free metal, P_b is the fraction of bound metal, $\Delta\nu_{1/2f}$ is the line width of free metal, and $\Delta\nu_{1/2b}$ is the line width of bound metal. $\Delta\nu_{1/2b}$ may be represented by $\Delta\nu_{1/2b} = \pi^{-1}(T_2 + k_{off}^{-1})^{-1}$.[33] When K_d of Ca^{2+} from CaM is 10^{-6} M[5], the exchange rate of Ca^{2+} from CaM can be roughly estimated as approximately 10^3 s^{-1}, which is comparable to that, 2.7×10^3 s^{-1}, obtained for *Tetrahymena* CaM.[9]

It was shown from the ^{43}Ca NMR spectral change caused by adding TFP that 2 mol of TFP is bound to 1 mol of CaM as has been described.[9,15] By the binding of TFP to CaM, the exchange rate will be markedly reduced by 1 order of magnitude (Figure 2B).[15] This phenomenon will be caused by a conformational change of the entire CaM molecule. It was found in this study that KCl does no affect the environmental change of the Ca^{2+} low-affinity site caused by the TFP binding. It seems unlikely that an environmental factor such as the symmetry around Ca^{2+} in CaM contributes to an appreciable extent to the line width of the metal NMR of the quadrupole metal cation bound to the macromolecule, since the asymmetrical contribution to the quadrupolar relaxation of exchangeable metal cation is usually very small and the quadrupolar coupling constant of the quadrupolar metal ion ionically interacting with the macromolecule may be constant compared with that of the aqueous ion.[15] It is also ruled out that spectral narrowing of the ^{43}Ca NMR of the Ca^{2+}–CaM solution by adding TFP is due to the increase of free Ca^{2+} content in the solution, since Ca^{2+} will be more tightly bound to CaM in the presence of TFP, conjectured from the fact that adding Ca^{2+} to Ca^{2+}-free CaM causes the TFP binding constant to increase by 10 times.[11,28]

Levin and Weiss[11] reported from equilibrium dialysis studies that CaM has two TFP binding sites, which are Ca^{2+} dependent, with K_d approximately 1 μM and nonspecific TFP binding sites, which are Ca^{2+} independent, with K_d approximately 5 mM. They also reported that TFP binding at the TFP concentration of more than 10^{-4} M is not Ca^{2+} dependent. Although TFP concentrations used in our ^{43}Ca NMR studies were up to 0.6 mM, the ^{43}Ca NMR studies described here decidedly indicate that 2 mol of TFP binds to 1 mol of CaM (Figure 2B), with K_d less than 0.2 mM. Thus, ^{43}Ca NMR changes caused by adding up to 0.6 mM TFP will reflect the TFP binding to the Ca^{2+}-dependent TFP high-affinity sites of CaM. Levin and Weiss[11] used 5 mM Tris, 1 mM Mg^{2+}, and 1.7 μM CaM solution to study the TFP binding to CaM. The experimental difference of our study from that of Levin and Weiss[11] may in part make interpretation of the TFP binding difficult. Gariépy and Hodges[29] reported that TFP concentration above the 1 mM range should not be used for spectral studies because of its self-aggregation.

^{25}Mg NMR. The excess relaxation rate increase of the ^{25}Mg NMR of free Mg^{2+} caused by adding CaM (Figure 3A) was more marked than that observed for the ^{43}Ca NMR of free Ca^{2+} by CaM (Figure 2A). The K_d value for Mg^{2+} from its binding site(s) in CaM is approximately 10^{-5}–10^{-4} M.[5] Thus, the exchange rate of Mg^{2+} from CaM will be more than that (10^3 s^{-1}) of Ca^{2+} from its low-affinity sites of CaM by 1 order of magnitude or more. The faster exchange rate of Mg^{2+} from CaM may cause more marked line broadening of the ^{25}Mg NMR compared with that of the ^{43}Ca NMR.

It has not been well established hitherto whether Mg^{2+} binds exactly to the Ca^{2+} binding sites in CaM or not. However, it seems very likely that ^{25}Mg NMR may reflect the binding of Mg^{2+} to the Ca^{2+} binding site in CaM, since the titration study by adding Ca^{2+} (Figure 4A) is indicative of the increase of free Mg^{2+} by its substitution. In this study, it became clear that Mg^{2+} decidedly binds to the Ca^{2+} binding site(s) in CaM. Conformational change or activation of CaM caused by Mg^{2+} is smaller than that by Ca^{2+}, Mn^{2+}, or Zn^{2+}.[5-10,14] Thus, Mg^{2+} will not cause a specific conformational change of CaM, which is necessary for specific CaM–TFP or CaM–enzyme interaction.

The line width of the ^{25}Mg NMR of the Mg^{2+}–CaM solution was decreased by adding TFP. Thus, it is shown here that TFP binds to CaM in the presence of Mg^{2+}.[14] However, the line width decrease of ^{25}Mg NMR of the Mg^{2+}–CaM solution caused by adding TFP was not so marked as that of ^{43}Ca NMR and occurred at [TFP]/[CaM] = 1 (Figure 3B). Thus, it was suggested that TFP binds to a site of CaM that is different from that in the

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Ca^{2+}–CaM solution and/or that TFP does not change the environment of the Mg^{2+} binding site in CaM so markedly as that of the low-affinity site for Ca^{2+} in CaM. Probably this is due to the fact that Mg^{2+} does not casue a specific conformation change of CaM that is necessary for the specific TFP–CaM interaction as mentioned earlier. Specific induction fo the hydrophobic character of CaM by Ca^{2+} should be noiced here. ^{113}Cd NMR changes for Cd^{2+} bound at low-affinity sites for Cd^{2+} (probably the same as those for Ca^{2+}) caused by adding TFP were more marked than those at high-affinity sites for Cd^{2+} (probably the same as those for Ca^{2+}).[28]

Adding KCl to the Mg^{2+}–CaM solution decreased the line width of the ^{25}Mg NMR of the solution (Figure 4B). Hitherto, we cannot conclude whether K^+ is substituted for Mg^{2+} in the Mg^{2+}–CaM solution of KCl markedly changes the conformation of CaM, leading to the ^{25}Mg NMR change. The ^{25}Mg NMR line width of the Mg^{2+}–CaM–KCl was further decreased by adding Ca^{2+}. It is suggested that Ca^{2+} binds to the Mg^{2+} binding site of CaM in the KCl solution.

The relatively small change of the ^{25}Mg NMR of the Mg^{2+}–CaM–TFP solution compared with that of the Mg^{2+}–CaM solution was observed by adding KCl (Figure 5A). KCl does not influence the induced CD spectra of the Mg^{2+}–CaM–TFP solution so markedly as that observed for other divalent metal–CaM–TFP solutions.[14] Thus, the $^{21}{}^{5}Mg$ NMR result is in accordance with the finding observed with the induced CD spectra of the Mg^{2+}–CaM–TFP solution. The line width decrease by adding Ca^{2+} to the Mg^{2+}–CaM–TFP solution (Figure 5C) is less marked than that observed by adding Ca^{2+} to the Mg^{2+}–CaM or to Mg^{2+}–CaM–TFP–KCl solutions (Figures 4A and 5B). Thus, Ca^{2+} seems to bind to the Mg^{2+} binding site in the Mg^{2+}–CaM–TFP–KCl solution (Figure 5B), while Ca^{2+} may in part bind to the Mg^{2+} binding site in the Mg^{2+}–CaM–TFP solution under these conditions.

^{67}Zn NMR. The line width increase of ^{67}Zn NMR of free Zn^{2+} caused by adding CaM was much more marked than that observed for Ca^{2+} or Mg^{2+} in terms of ^{43}Ca or ^{25}Mg NMR. From induced CD spectral studies[14] it was estimated that the K_d value of Zn^{2+} from CaM will be 10^{-4}–10^{-3} M. This relatively high K_d value of Zn^{2+} compared with that of Ca^{2+} or Mg^{2+} will cause a faster exchange rate (approximately 10^5–10^6 s^{-1}) of Zn^{2+} from CaM, which may lead to more marked line broadenings of the ^{67}Zn NMR of the Zn^{2+}–CaM solutions.

It was first found in this paper that the binding behavior of Zn^{2+} to CaM in HEPES–Na^+ is different from that in HEPES–K^+ (Figure 6A). It seems likely that K^+ inhibits in part the Zn^{2+} binding to CaM rather than lowering the exchange rate of Zn^{2+} from CaM. The line width of the ^{67}Zn NMR of the Zn^{2+}–CaM–HEPES–K^+ solution was decreased by adding Ca^{2+}, while that of the Zn^{2+}–CaM–HEPES–Na^+ solution was not (Figure 7A). Thus, the Zn^{2+} binding site of CaM may be the same as the Ca^{2+} binding site of CaM in HEPES–K^+ solution. Therefore, it was suggested that the Zn^{2+} binding site of CaM is the same as the K^+ binding site and the Ca^{2+} binding site. It was suggested from ^{113}Cd NMR study[10] that Zn^{2+} binds at Ca^{2+} high-affinity sites in distilled water. The Zn^{2+} binding site of the CaM–HEPES–Na^+ solution may be different from the K^+ binding site of CaM, since adding excess KCl did not change the ^{67}Zn NMR of the Zn^{2+}–CaM–HEPES–Na^+ (Figure 7B) and the Zn^{2+}–CaM–TFP–HEPES–Na^+ (Figure 8B) solution. The Zn^{2+} binding sites of the CaM–HEPES–Na^+ and CaM–TFP–HEPES–Na^+ solutions may be also different from the Ca^{2+} binding sites of the same solutions, since ^{67}Zn NMR of the same solutions was not changed by adding excess Ca^{2+} (Figure 7A and 8C). The difference in metal NMR behavior between Na^+ and K^+ may be ascribed to that of the ionic radii between Na^+ (0.95 Å) and K^+ (1.33 Å).[30]

The environment of Zn^{2+} in CaM was only slightly influenced by TFP as can be seen in Figure 8A. The environmental change of Zn^{2+} in the CaM–HEPES–Na^+ solution caused by adding TFP may be much smaller than that observed for Ca^{2+}–CaM caused by adding TFP with ^{43}Ca NMR.

^{39}K NMR. The ^{39}K NMR spectral change of free K^+ caused by adding CaM was very small compared with the changes in the ^{43}Ca, ^{25}Mg, and ^{67}Zn NMR of free-metal cations by adding CaM, suggesting that the binding ability of K^+ to CaM is very low. K_d of K^+ from CaM was estimated to be 10^{-2} M from induced CD[14] and ^{25}Mg NMR (Figures 4 and 5). Thus, the exchange rate will be in a very fast exchange region, which may not result in heavy line broadening of the ^{39}K NMR and may not show temperature dependence of the line width. It may be possible that K^+ interacts with the Ca^{2+} binding site of CaM and CaM–TFP, since addition of Ca^{2+} to the K^+–CaM or K^+–CaM–TFP solutions decreased the line widths of the solutions (Figure 9B,D). K^+ may bind preferentially to the Ca^{2+} high-affinity sites, since the Ca^{2+} low-affinity sites are not affected by KCl in terms of ^{43}Ca NMR. The decreases of the ^{39}K NMR for K^+ (10 mM)–CaM (0.26 mM) and K^+ (10 mM)–CaM (0.26 mM)–TFP (0.9 mM) solutions caused by adding Ca^{2+} were saturated at $[Ca^{2+}]/[CaM]$ ratios of 2 and 1, respectively. The number of Ca^{2+} high-affinity sites of K^+–CaM and K^+–CaM–TFP solutions may be 2 and 1, respectively. Another alternative interpretation may be possible for the decrease of the line width of the ^{39}K NMR of K^+–CaM by adding Ca^{2+}. That is, the affinity of Ca^{2+} to K^+–CaM was increased by the presence of TFP. This result is compatible with that implied from the ^{43}Ca NMR study.

The slight decrease of the ^{39}K NMR line width of K^+ (10 mM)–CaM (0.26 mM) was also observed by adding 0.3–0.4 mM TFP (Figure 8C), suggesting that TFP will bind to K^+–CaM with stoichiometry $[TFP]/[CaM] = 1$ as has been implied from the ^{19}F NMR study.[13,25] Here again, it is shown that CaM does not necessitate Ca^{2+} for TFP binding.[13,14,29]

Conclusion. As concluding remarks, the following were suggested from ^{43}Ca NMR spectra: (1) The environment of Ca^{2+} bound to the Ca^{2+} low-affinity sites is not influenced by KCl. (2) The environment of Ca^{2+} bound to the Ca^{2+} low-affinity-sites is markedly changed by adding TFP. From ^{25}Mg NMR spectra the following werre observed: (1) The binding site(s) for Mg^{2+} in CaM may be the same as that for Ca^{2+}. (2) Mg^{2+} may not cause the specific conformational change of CaM that is necessary for the specific TFP–CaM interaction. From ^{67}Zn NMR spectra the following were observed: (1) The Zn^{2+} binding site(s) in CaM is the same as the K^+ binding site(s) in CaM. (2) Ca^{2+} can bind to the Zn^{2+} binding site(s) in CaM in the presence of K^+. (3) The binding site(s) of Na^+ in CaM is different from that of K^+. From ^{39}K NMR spectra the following were observed: (1) K^+ may interact with the Ca^{2+} binding sites in CaM and in CaM–TFP.

Subtle but important differences in structure between Ca^{2+} and other metal cations such as Mg^{2+} and lanthanides (Ln^{3+}) have been excellently reviewed.[32,35] From laser Raman spectroscopy of CaM solutions, it was suggested[34] that local conformational changes in CaM are induced by both Ca^{2+} and Mg^{2+}, but a conformational change involving the peptide backbone is caused only by Ca^{2+} addition. Induced CD study of the CaM–TFP soluiton indicates that Mg^{2+} does not cause a specific CaM–TFP interaction as Ca^{2+} does.[14] Similar results of the bindings of Ca^{2+} and other metal cations to other Ca^{2+}-binding proteins have been reported. For example, binding constants of Mg^{2+} and Zn^{2+} to a Ca^{2+}-binding protein, elastase, are much lower than that of Ca^{2+} in terms of luminescence spectra by using the terbium cation.[36] Our metal NMR studies suggested that the bivalent (such as Mg^{2+} and Zn^{2+}) and monovalent (such as K^+) metal cations can actualy

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bind to the specific Ca^{2+}-binding site(s) of CaM under our experiiental conditions, although ionic valence numbers, effective ionic radii, binding constants, and coordination structures of these cations are different from each other.[32,35] The very specific role of Ca^{2+} in intracellular Ca^{2+}-related functions may be due to its ability to cause hydrophobic character of Ca^{2+}-dependent proteins. Very variable bond lengths and coordination numbers of Ca^{2+} compared with other metal cations may be related to the specific character of Ca^{2+}.[30,32,35] It should be emphasized here that the quadrupole metal NMR method is quite useful for obtaining individual information on each metal binding site in macromolecules.

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Registry No. ^{43}Ca, 14333-06-3; ^{25}Mg, 14304-84-8; ^{67}Zn, 14378-34-8; ^{39}K, 14092-91-2; Ca, 7440-70-2; Mg, 7439-95-4; Zn, 7440-66-6; K, 7440-09-7; TFP, 117-89-5.

Supplementary Material Available: A figure showing temperature dependences of ^{43}Ca, ^{25}Mg, and ^{67}Zn NMR line widths for Ca^{2+}–, Mg^{2+}–, and Zn^{2+}–CaM solutions (2 pages). Ordering information is given any current masthead page.

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Synthesis and Molecular Structure of Five-Coordinated Spirocyclic Anionic Silicates Containing *tert*-Butyl Groups. Hydrogen-Bonding Effects[1,2]

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The new five-coordinated anionic silicates $[((t\text{-Bu})_2C_6H_2O_2)_2SiPh][Et_3NH]$ (**1**), $[(C_6H_4O_2)_2SiPh][Et_3NH]$ (**2**), and $[(C_{10}H_6O_2)_2Si\text{-}t\text{-Bu}][Et_4N]$ (**3**) were synthesized and their X-ray structures obtained. ^1H, ^{29}Si, and ^{13}C NMR spectral data also are reported. The structures are displaced 29.0% for **1**, 59.4% for **2**, and 80.3% for **3** from the trigonal bipyramid toward the rectangular pyramid (based on unit bond distances). It is concluded that hydrogen bonding between the ammonium cation and oxygen atoms of the spirocyclic framework, e.g. in **2**, causes displacement of the structural form toward the rectangular pyramid compared to related structures lacking this hydrogen-bonding possibility. It is felt that the use of *tert*-butyl ring substituents in **1** shields the silicon center from the hydrogen-bonding effect of the cation and, hence, accounts for the near-trigonal-bipyramidal geometry observed. Details of this form of the distortion coordinate are presented and shown to be closely related to nonrigid phosphoranes and arsoranes. **1** crystallizes in the orthorhombic space group $P2_12_12_1$ with a = 11.849 (1) Å, b = 17.040 (2) Å, c = 19.857 (3) Å, and Z = 4. **2** crystallizes in the monoclinic space group $P2_1/n$ with a = 10.492 (2) Å, b = 21.104 (5) Å, c = 10.598 (1) Å, β = 98.92 (1)°, and Z = 4. **3** crystallizes in the monoclinic space group $P2_1/n$ with a = 9.678 (2) Å, b = 25.368 (7) Å, c = 12.909 (3) Å, β = 108.18 (2)°, and Z = 4. The final conventional unweighted residuals are 0.065 (**1**), 0.042 (**2**), and 0.081 (**3**).

Introduction

We have shown that the molecular structures of pentacoordinated anionic silicon complexes[3,4] follow the same type of distortion coordinate as observed for isoelectronic phosphoranes,[5,7] i.e., the Berry pseudorotational coordinate that connects the ideal trigonal bipyramid (TBP) with a square or rectangular pyramid (RP). The latter coordinate has been supported as the principal one accounting for NMR ligand-exchange phenomena in a wide variety of pentacoordinated phosphorus compounds.[8,9] This same coordinate is followed by the structures of arsoranes[1,10] and five-coordinated germanium compounds so far examined.[11] In all of these cases, a trans basal angle, θ, near 150° is indicated for the geometry of the "ideal" square pyramid. This contrasts with that found for five-coordinated transition-metal derivatives that show θ angles varying from 140 to 175°.[12,13]

The general features that stabilize the normally higher energy square pyramid for phosphoranes[6,7,14] are found to apply equally well to the less well-studied five-coordinated silicon[3] compounds. Most of the observed structural distortions are consistent with expectations from substituent effects.[3,4,6,7,15] However, unlike the molecular phosphoranes, the isoelectronic five-coordinated silicates have additional complicating features owing to the saltlike character of the complexes and the presence of hydrogen bonding between hydrogen-containing cations and oxygen atoms of the silicon anion.

In this paper, an attempt is made to evaluate the role of these two lattice effects in influencing the geometrical distortion of pentacoordinated silicon compounds. The compounds chosen for study are $[((t\text{-Bu})_2C_6H_2O_2)_2SiPh][Et_3NH]$ (**1**), $[(C_6H_4O_2)_2SiPh][Et_3NH]$ (**2**), and $[(C_{10}H_6O_2)_2Si\text{-}t\text{-Bu}][Et_4N]$ (**3**).

The first structure of an anionic silicate compound that was established was an X-ray study of **4** performed in 1968.[16] The geometry is displaced approximately one-third the way from the TBP.[3] The numbers in parentheses below the formula representations are the percent displacement along the Berry coordinate from the TBP toward the RP calculated by a dihedral angle

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