Unless indicated otherwise, all steps proceed with rate constant k'.

Given the symmetry of the above scheme whereby all species in any column are equivalent, the differential equations can be integrated similarly to Scheme II to yield the solution given in the text:

$$\frac{[P^*S_1S_2S_3S_4]}{[P_0]} = 1 - \frac{4k_c}{k_c - k'}e^{-k't} + \frac{6k_c}{k_c - 2k'}e^{-2k't} - \frac{4k_c}{k_c - 3k'}e^{-3k't} + \frac{k_c}{k_c - 4k'}e^{-4k't} - \frac{24k'^4}{(k_c - k')(k_c - 2k')(k_c - 3k')(k_c - 4k')}e^{-k_ct}$$

It is instructive to consider the more general case of a protein unit with n subunits each having an equivalent thiol ester that must be cleaved in order to trigger a conformational change in the protein unit. Integration of the differential equations yields

$$\frac{[P^*S_1S_2...S_n]}{[P_0]} = 1 - \frac{nk_c}{k_c - k'}e^{-k't} + \frac{n(n-1)k_c}{2!(k_c - 2k')}e^{-2k't} - \frac{n(n-1)(n-2)k_c}{3!(k_c - 3k')}e^{-3k't} + ... + (-1)^{n-1}\frac{nk_ce^{-(n-1)k't}}{k_c - (n-1)k'} + (-1)^n\frac{k_ce^{-nk't}}{k_c - nk'} + (-1)^{n+1}\frac{n!k'^ne^{-k_ct}}{(k_c - k')(k_c - 2k')...(k_c - nk')}$$

The first $(n + 1)$ terms contain the binomial coefficients as do the number of species in each column of the mechanism [see model 2 (Scheme II) and model 3 above]. For all mechanisms, summing all sulfhydryl-containing species gives

$$\frac{[S]_{total}}{n[P_0]} = 1 - e^{-k't}$$

verifying their kinetic equivalence, as assumed.

Nuclear Magnetic Resonance Studies on Calmodulin: Ca^{2+}-Dependent Spectral Change of Proteolytic Fragments†

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ABSTRACT: Proton magnetic resonance spectroscopy was performed in order to study the effect of calcium on the solution conformation of calmodulin with four proteolytic fragments. Two characteristic high field shifted phenylalanines of Ca^{2+}-free calmodulin were found to be located in domain I and/or domain II. In the Ca^{2+}-saturated state, these two phenylalanines also give resonances at higher fields. Another high field shifted phenylalanine appears and is found to be located in domain III or domain IV. It was demonstrated that the interaction between domains I and II and the interaction between domains III and IV are of importance for stabilization of the native structure but the interaction between the N-terminal-half region (domains I and II) and the C-terminal-half region (domains III and IV) was not clarified. Ca^{2+}-dependent slow-exchange behavior of ϵ and δ protons of tyrosine-138 [Ikura, M., Hiraoki, T., Hikichi, K., Mikuni, T., Yazawa, M., & Kagi, K. (1983) *Biochemistry 22*, 2573] was confirmed. The conformational transition between the Ca^{2+}-free and -bound states occurs at a rate of more than 300 s^{-1} in the N-terminal-half fragment, while the transition of the C-terminal-half fragment is slower than 50 s^{-1}.

Calmodulin (CaM)[1] and troponin C (TnC) are homologous Ca^{2+} binding proteins. The former serves as a Ca^{2+}-dependent activator or modulator of numerous enzymes, and the latter regulates muscle contraction in a Ca^{2+}-dependent manner [for a review, see Klee & Vanaman (1982)]. Both proteins consist of four Ca^{2+}-binding domains, numbered as I, II, III, and IV starting from the N terminal. Each domain is made of about 35 amino acid residues, and the sequences of each domain are homologous to one another. The structure of each domain has been considered to be similar to the EF-hand structure of parvalbumin crystal reported by Kretsinger & Barry (1975).

A number of spectroscopic studies show that binding of Ca^{2+} to CaM and to TnC causes considerable conformational changes [for a review, see Klee & Vanaman (1982)]. Because the difference in function between CaM and TnC should be related to the difference in structure, it is of interest to study the conformation of CaM and the conformational change induced by Ca^{2+} binding in detail as compared to TnC.

In earlier proton NMR studies on CaM (Seamon, 1980; Krebs & Carafoli, 1982) and also in our succeeding studies (Ikura et al., 1983a,b), assignments of resonances appearing in the aromatic and high-field methyl regions were made, and the Ca^{2+}-dependence of the spectra was investigated. Because the resonances were assignable only to residues in the C-terminal-half region (domains III and IV), little information about the N-terminal-half region (domains I and II) was obtained.

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[1] Abbreviations: CaM, calmodulin; TnC, troponin C; NMR, nuclear magnetic resonance; CD, circular dichroism; F1, cyanogen bromide fragment containing domain I (residues 1–36); F4, tryptic fragment containing domain IV (residues 107–148); F12, tryptic fragment containing domains I and II (residues 1–75); F34, tryptic fragment containing domains III and IV (residues 78–148); TCA, trichloroacetic acid; TSP, (trimethylsilyl)propionic-d_4 acid; Tml, N^{ϵ},N^{ϵ},N^{ϵ}-trimethyllysine; NOE, nuclear Overhauser enhancement.

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Proton NMR studies on proteolytic fragments of TnC revealed that the native structure is preserved to a great extent in the fragments, and assignments of resonances to specific residues were achieved by spectral comparison between the intact TnC and the fragments (Evans et al., 1980; Leavis et al., 1980). Drabikowski et al. (1982) studied CD and tyrosine fluorescence of CaM fragments and showed that the TR1-C fragment (consisting of domains I and II) and the TR2-C fragment (consisting of domains III and IV) retain most of the secondary structure that possesses in the intact CaM. Thus, proton NMR studies on the fragments of CaM will promise to give more detailed information about assignments and structure, especially of the N-terminal-half region.

In this paper, we present proton NMR spectra of four CaM fragments: F1 (1st–36th residues including domain I), F4 (107th–148th residues including domain IV), F12 (1st–75th residues including domains I and II), and F34 (78th–148th residues including domains III and IV).[2] The spectra obtained for these fragments are compared with those of the intact CaM. The Ca^{2+}-induced conformational change of the intact protein will be discussed in more detail.

Experimental Procedures

Scallop testis CaM was isolated by the method described by Yazawa et al. (1978). Fragments of CaM were prepared by using cyanogen bromide (for F1) or trypsin (for F4, F12, and F34) as described by Toda et al. (1981). The purity was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. All samples were decalcified by the TCA precipitation method (Yazawa, 1980). The amount of residual Ca^{2+} was less than 0.1 mol/mol of protein for CaM and F34 and it was about 0.2 mol/mol for F12 as measured by a Hitachi 208 atomic absorption spectrometer. Proteins were lyophilized in D_2O to replace exchangeable hydrogen with deuterium. Solutions of 10 mg of proteins in 0.4 mL of D_2O containing 0.2 M KCl were used for NMR measurements.

The Ca^{2+} titration was made by adding successively 2 μL of 96 mM $CaCl_2$ to the samples. The pH titration was performed by adding 4% KOD or 4% DCl. pH values are not corrected for the deuterium isotope effect.

Proton NMR measurements were carried out at a frequency of 400 MHz on a JEOL FX-400 spectrometer operating in a pulse Fourier-transform mode with quadrature detection. Spectra were usually obtained from accumulation of 128–512 free-induction decays after each 45° pulse (5 μs), repeated every 4 s and observed over a frequency range of 4000 Hz with 16K data points. Chemical shifts were measured in parts per million (ppm) from the internal standard of (trimethylsilyl)-propionic-d_4 acid (TSP). The residual HDO peak was suppressed by the gated decoupling method (Hoult & Richards, 1975). A part of the NMR measurements was made at a frequency of 500 MHz on a JEOL GX-500 spectrometer. The conditions of the experiment were same as those of the 400-MHz experiments except for the spectral range of 5000 Hz.

The numbering of amino acid residues of fragments refers to that of intact CaM (Toda et al., 1981).

Results

Ca^{2+}-Induced Spectral Change of F1 and F4 Fragments. Figure 1 shows the aromatic region of the 400-MHz proton NMR spectra of F1 fragment in the absence (A) and the presence (B) of Ca^{2+}. F1 contains only three phenylalanines

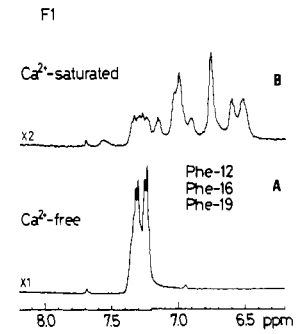

FIGURE 1: Aromatic region of the 400-MHz ^{1}H NMR spectra of fragment F1: (A) Ca^{2+}-free state, $[Ca^{2+}]$ = 0 mM; (B) Ca^{2+}-saturated state, $[Ca^{2+}]$ = 46 mM; [F1] = 2.6 mM, 0.2 M KCl, pH 8.9, 22 °C.

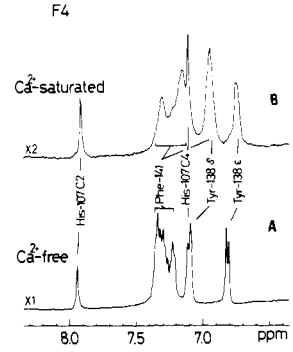

FIGURE 2: Aromatic region of the 400-MHz ^{1}H NMR spectra of fragment F4: (A) Ca^{2+}-free state, $[Ca^{2+}]$ = 0 mM; (B) Ca^{2+}-saturated state, $[Ca^{2+}]$ = 112 mM; [F4] = 4.1 mM, 0.2 M KCl, pH 6.7, 22 °C.

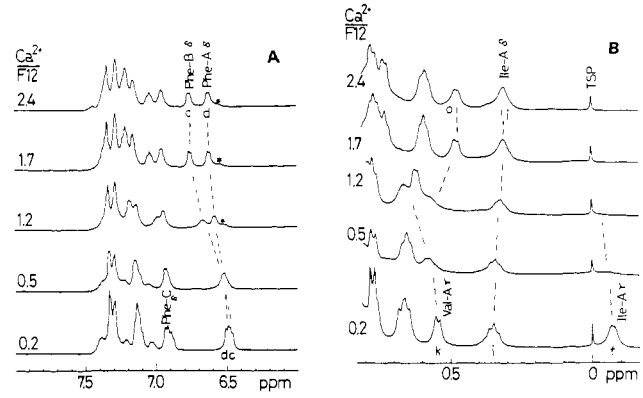

FIGURE 3: The 400-MHz ^{1}H NMR spectra of fragment F12 as a function of calcium content: (A) aromatic region; (B) high-field methyl region; [F12] = 3.0 mM, 0.2 M KCl, pH 7.9, 22 °C. An asterisk (*) indicates an impurity.

(12, 16, and 19) as aromatic residues. It is seen in Figure 1A that in the absence of Ca^{2+}, resonances of ring protons of all phenylalanines appear in the vicinity of 7.3 ppm where a free amino acid phenylalanine normally exhibits the resonance. The results suggest that F1 has little tertiary structure in the absence of Ca^{2+}. Two small singlet peaks appearing at 6.95 and 7.69 ppm are probably due to His-107 of other fragments contaminating the sample. The addition of Ca^{2+} causes a change in the spectra as shown in Figure 1B. Some Phe peaks shift high field markedly, suggesting that F1 forms a tertiary structure upon the addition of Ca^{2+}.

Figure 2 shows the aromatic region of the spectra of F4 in the absence (A) and the presence (B) of Ca^{2+}. F4 contains one histidine (His-107), one tyrosine (Tyr-138), and one phenylalanine (Phe-141). The resonances of His-107, Tyr-138, and Phe-141 appear at normal chemical shift positions in the absence of Ca^{2+} (Figure 2A), suggesting an unfolded structure of F4. Upon addition of Ca^{2+} (Figure 2B), Tyr-138 δ and ϵ,

[2] F4, F12, and F34 in the present study correspond respectively to TR3-E, TR1-C, and TR2-C in the previous study (Drabikowski et al., 1982).

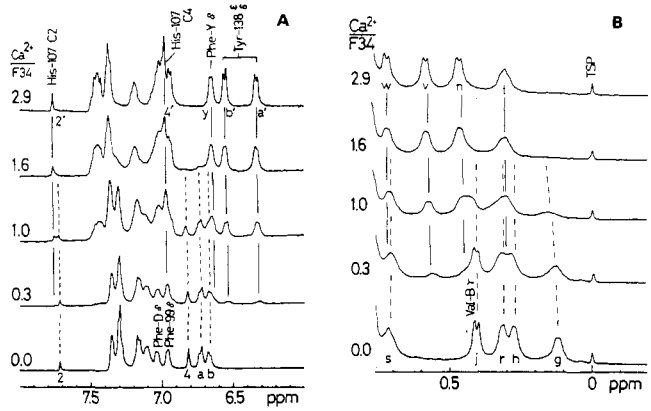

FIGURE 4: The 400-MHz ^1H NMR spectra of fragment F34 as a function of calcium content: (A) aromatic region; (B) high-field methyl region; [F34] = 3.0 mM, 0.2 M KCl, pH 8.3, 22 °C.

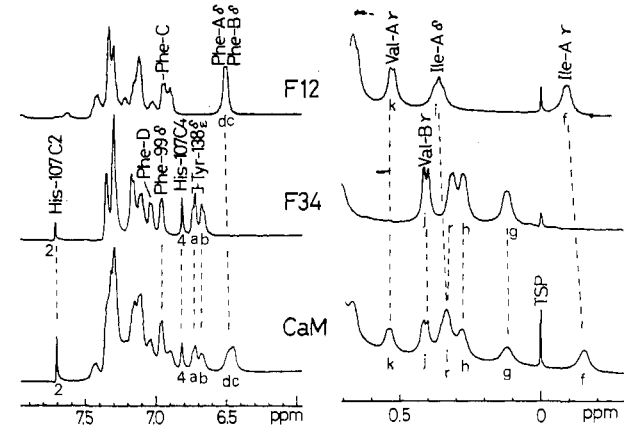

FIGURE 5: The 400-MHz ^1H NMR spectral comparison of intact CaM with fragments F12 and F34 in the Ca^{2+}-free state: (left) aromatic region; (right) high-field methyl region; (bottom) [CaM] = 1.5 mM, pH 8.3; (middle) [F34] = 3.0 mM, pH 8.3; (top) [F12] = 3.0 mM, pH 5.4. All included 0.2 M KCl, and the temperature was 22 °C.

as well as Phe-141 δ, resonances shift high field. This indicates the formation of a tertiary structure of F4.

Ca^{2+} Titration of F12 Fragment. Figure 3A shows the aromatic region of the proton NMR spectra of F12 fragment as a function of the molar ratio of Ca^{2+} to F12. The aromatic spectral region comprises resonances from ring protons of five Phe's (12, 16, 19, 65, and 68). In the absence of Ca^{2+}, some Phe peaks appear at extremely high fields, suggesting the existence of a tertiary structure in F12 in contrast to an unfolded structure of Ca^{2+}-free F1 and F4 fragments.

The most high field shifted Phe resonances, designated as peaks c and d in Figure 3A, are two doublet peaks of δ protons of two Phe's. Upon the addition of Ca^{2+}, peaks c and d shift downfield continuously, but the peaks still remain at higher fields as compared to other aromatic resonances. In the Ca^{2+}-free state, peak c appears at higher fields than peak d, but in the Ca^{2+}-saturated state, they appear in reverse order. At intermediate Ca^{2+} contents, peak c crosses peak d, and peak c broadens more significantly than peak d. The crowded region of 6.9–7.5 ppm is also affected by the addition of Ca^{2+}.

Figure 3B shows the high-field methyl region at various Ca^{2+} contents. Four Ile's (9, 27, 52, and 63), two Val's (35 and 55), six Leu's (4, 18, 32, 39, 48, and 69), eight Thr's (5, 26, 28, 29, 34, 44, 62, and 70), and four Ala's (1, 10, 15, and 57) are expected to give methyl resonances in this region. Two doublet peaks f and k in the Ca^{2+}-free state shift downfield upon addition of Ca^{2+} and disappear in the Ca^{2+}-saturated state. The triplet peak i shifts slightly high field and remains at the most high field position in the Ca^{2+}-saturated state. The triplet methyl peak i is assigned to Ile δ-methyl protons, and other doublet methyl resonances are assigned to methyl protons of any of Ile, Leu, Val, Thr, or Ala.

The low field shifted α-methine resonance that was observed previously for the intact CaM (Ikura et al., 1983b) is also found in the spectrum of F12 (data not shown) but consists of two peaks. Titration of the resonance with calcium results in a continuous change in chemical shift from 5.55 to 5.35 ppm in a similar manner as in the case of intact CaM.

Ca^{2+} Titration of F34. The aromatic region of the proton NMR spectra of F34 is shown in Figure 4A as a function of the molar ratio of Ca^{2+} to F34. All peaks in this region are due to ring protons of Phe-89, Phe-92, Phe-99, Phe-141, His-107, and Tyr-138. The assignments of His-107 and Tyr-138 were made by the pH titration experiment. The resonances of C2 and C4 protons of His-107 shift high field with increasing pH, and the titration curve of the His-107 chemical shifts gives $pK_a = 6.4 \pm 0.2$ both in the absence and the presence of Ca^{2+}. The resonances of δ and ε protons of Tyr-138 (a and a′ for δ; b and b′ for ε) also shift high field with increasing pH. The pK_a value of Tyr-138 is 12.1 ± 0.2 both in the absence and the presence of Ca^{2+}.

In the course of Ca^{2+} titration, Tyr-138 δ and ε resonances [peaks a and b in Figure 4A (bottom)] do not move but decrease in intensity in parallel with increases of two new resonances (peaks a′ and b′), which correspond to those in the Ca^{2+}-saturated state [Figure 4A (top)]. The resonances of His-107 C2 and C4 protons (peaks 2 and 2′ for C2; peaks 4 and 4′ for C4) show a similar spectral behavior as Tyr-138 δ and ε resonances. A new doublet peak (y) due to one of four Phe's appears at a relatively high-field position (6.63 ppm) and increases in intensity upon the addition of Ca^{2+}.

Figure 4B shows the high-field methyl region at various Ca^{2+} contents. The methyl resonances in this region are contributed from any of four Ile's (85, 100, 125, and 130), five Val's (91, 108, 121, 136, and 142), three Leu's (105, 112, and 116), or five Ala's (88, 102, 103, 129, and 147). With increasing Ca^{2+} content, four doublet methyl resonances (peaks g, h, r, and j) and a triplet methyl resonance (peak s) decrease in intensity, while three doublet methyl resonances (peaks u, v, and w) and a triplet methyl resonance (peak t) newly appear and increase in intensity.

The strongest singlet peak of Tml-115 $N^{\epsilon},N^{\epsilon},N^{\epsilon}$-trimethyl protons was found to appear at 3.13 and 3.11 ppm in Ca^{2+}-free and -saturated states, respectively (data not shown). At intermediate Ca^{2+} contents, Tml-115 shows two peaks simultaneously in the same manner as Tyr-138 and His-107.

Discussion

As is the case of TnC fragments containing a single Ca^{2+} binding domain, that is, CB8 (domain II of TnC), CB9 (domain III of TnC), and TH2 (domain IV of TnC) (Leavis et al., 1982), F1 and F4 fragments of CaM have little tertiary structure in the absence of Ca^{2+} and form a tertiary structure upon the addition of Ca^{2+}. On the other hand, fragments F12 and F34 with two Ca^{2+} binding domains have well-defined structure both in the absence and the presence of Ca^{2+}.

Figure 5 shows the spectral comparison of F12, F34, and intact CaM in the Ca^{2+}-free state. Chemical shifts of His-107 and Tyr-138 of Ca^{2+}-free F34 are identical with those reported with Ca^{2+}-free intact CaM (Seamon, 1980; Krebs & Calafori, 1982; Ikura et al., 1983a). In our previous study on intact CaM (Ikura et al., 1983a), first-stage assignments were made for resonances of seven Phe's (labeled with A–G), two Val's (A and B), and one Ile (A). Phe-E was assigned to Phe-99

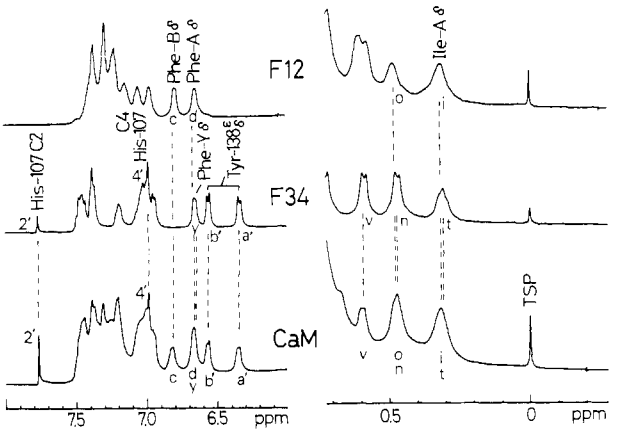

FIGURE 6: The 400-MHz ^{1}H NMR spectral comparison of intact CaM with fragments F12 and F34 in the Ca^{2+}-saturated state: (left) aromatic region; (right) high-field methyl region; (bottom) [CaM] = 1.5 mM, pH 8.3; (middle) [F34] = 3.0 mM, pH 5.4; (top) [F12] = 3.0 mM, pH 5.4. All included 6–8 mM $CaCl_2$ and 0.2 M KCl, and the temperature was 22 °C.

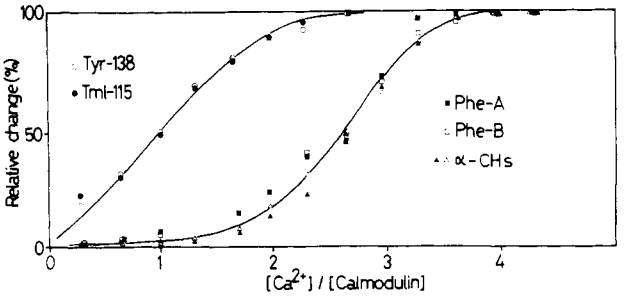

FIGURE 7: Relative changes in peak intensity and chemical shift as a function of molar ratio of added Ca per calmodulin. [CaM] = 1.5 mM; [KCl] = 0.2 M, pH 9.8 [the relatively high pH conditions are required for good peak separation of Phe-A and Phe-B; the titration curve was not essentially affected by pH in the range 7.8–9.8]; 22 °C. Data points were obtained from the 500-MHz ^{1}H NMR spectrum of intact CaM on the succesive addition of $CaCl_2$. Tyr-138 δ ring protons and Tml-115 N^{ϵ},N^{ϵ},N^{ϵ}-trimethyl protons are monitored by peak intensity. Phe-A δ ring protons, Phe-B δ ring protons, and the low field shifted α-methines are plotted by chemical shift.

by means of a spectral comparison between scallop testis and pig brain CaM's. Resonances of Phe-E, Phe-D, and Val-B appear in the spectrum of F34 at the same chemical shift position as those in the spectrum of the intact CaM; on the other hand, those of Phe-A, Phe-B, Phe-C, Val-A, and Ile-A appear in the spectrum of F12. The simple superposition of the two spectra of F12 and F34 is quite similar to the spectrum of intact CaM. Thus, resonances of F12 and F34 can be assigned by comparison with intact CaM. We labeled several peaks by the same symbol as used in the previous paper (Ikura et al., 1983b) in case that resonances of F12 or F34 correspond to those of CaM.

F12 shows five singlet methyl resonances arising from the N-terminal acetyl and four methionines at positions of 35, 51, 71, and 72. F34 exhibits four singlet methyl resonances of four methionines at positions 109, 124, 144, and 145. Both fragments lack Met-76. A high field shifted singlet methyl peak observed at 1.46 ppm for Ca^{2+}-free F12 is likely to correspond to the peak at 1.47 ppm found in the Ca^{2+}-free intact CaM (data not shown). Other singlet methyl resonances of the fragments appear in a range of 1.8–2.2 ppm as similarly as those of the intact CaM.

In the presence of Ca^{2+}, the overall spectral feature of the intact CaM is also similar to the superposition of F12 and F34 spectra as shown in Figure 6. In particular, aromatic and high-field methyl regions are to be noticed. The resonances of Tyr-138 and His-107 appear at the same chemical shift positions for both F34 and intact CaM. The high-field Phe peak at 6.67 ppm in intact CaM is found to consist of two Phe residues: one is Phe-A δ protons of F12, and the other Phe belongs to F34, which is now referred to as Phe-Y. The high field shifted Phe resonance observed at 6.82 ppm in F12 appears also at the same chemical shift position as that observed in intact CaM. Titration of F12 with Ca^{2+} showed that the resonance corresponds to δ protons of Phe-B.

The results obtained here indicate that two characteristic high field shifted Phe's of Ca^{2+}-free intact CaM (Phe-A at 6.50 ppm and Phe-B at 6.46 ppm) (Seamon, 1980; Krebs & Calafori, 1982; Ikura et al., 1983a,b) are located in domain I and/or domain II. Ca^{2+}-saturated intact CaM has three high field shifted Phe's: two of them are Phe-A (6.68 ppm) and Phe-B (6.83 ppm), and one of them is Phe-Y (6.68 ppm) located in domain III or domain IV.

The present study on the fragments suggests that F12 and F34 assume quite similar tertiary structures as those of the corresponding domains of the intact CaM both in the absence and the presence of Ca^{2+}. On the other hand, structures of F1 and F4 fragments are different from those of intact CaM. Even at saturated Ca^{2+} content, F1 and F4 fragments do not retain similar tertiary structures as that of the intact CaM. Furthermore, Tyr-138 has a high pK_a value of about 12 for both intact CaM and F34, while Tyr-138 of F4 has a normal pK_a value of about 11 in the presence of Ca^{2+} (M. Ikura et al., unpublished results). These results suggest that the interaction between domain I and domain II and the one between domain III and domain IV are of importance for stabilization of the structure of the native protein. The importance of such interdomain interaction has been also proposed for TnC (Leavis et al., 1980). The difference between CaM and TnC is that in the absence of Ca^{2+}, the TR2-C fragment of TnC assumes no tertiary structure, while the F34 fragment of CaM retains the native structure.

We could not clarify the importance of the interaction between the N-terminal- and C-terminal-half domains for stabilization of the native tertiary structure of CaM. Small differences in spectra in the vicinity of 0.6 ppm between F12 and CaM presumably represent a difference in structure between the two.

Previous NMR studies on intact CaM (Seamon, 1980; Krebs & Calafori, 1982) suggest a two-step conformational transition accompanied by Ca^{2+} binding. In our previous paper (Ikura et al., 1983b), we classified resonances of native CaM into three groups with respect to the Ca^{2+}-dependent spectral change: resonances of the first group change in a range of 0–2 mol of Ca^{2+}/mol of CaM, those of the second group in a range of 2–4 mol/mol, and those of the third group in a range of 0–4 mol/mol. The classification should be modified now because previous peak assignments were corrected in the high-field aromatic region (6.3–6.9 ppm) and the high-field methyl region (−0.2 to 0.8 ppm). Phe-B, which was classified previously to group I, shows a remarkable change in a range of 2–4 mol/mol as well as Phe-A in the present study. It is more appropriate to classify resonances into two groups: the first group member changes in a range of 0–2 mol/mol, and the second group member alters mostly in a range of 2–4 mol/mol. In Figure 7, the relative changes in peak intensity and chemical shift of the 500-MHz proton NMR spectra of the intact CaM are plotted against the molar ratio of Ca^{2+} to CaM for Tyr-138, Tml-115, Phe-A, Phe-B, and the low field shifted α-methines. This figure clearly shows that there are two groups of resonances with respect to the Ca^{2+}-dependent conformational change. The resonances classified to the first

group are assigned to residues existing in domains III and IV and those of the second group to residues in domains I and II. It is natural to consider that the first group reflects the high-affinity domains and the second group the low-affinity domains. These results suggest that the conformational changes in the two separated domains of I and II (N-terminal half) and III and IV (C-terminal half) occur mostly as a consequence of the binding of Ca^{2+} to the respective domains.

It has been reported by Levine et al. (1977), Evans et al. (1980), and Hincke et al. (1981) that high field shifted Phe and Ile in the low-affinity domains I and II of TnC are purturbed only in a range of more than 2 mol of Ca^{2+}/mol of protein. High field shifted Phe's (Phe-A and Phe-B) existing in the domains I and II of intact CaM also change markedly in a range of 2–4 mol/mol. More detailed inspection of the titration curve of Figure 7 shows that a slight increase begins at 1 mol/mol. A high field shifted doublet methyl resonance of Ile-A, which is located in the domains I and II, is apparently perturbed in a range of 1–2 mol/mol, though perturbation could not be observed in a range more than 2 mol/mol because of the interruption of crowded methyl peaks. Such change in a range less than 2 mol/mol for CaM has not been reported for TnC. This difference between CaM and TnC is probably due to the difference in Ca^{2+} binding affinity; for CaM, K_a of the high- and low-affinity sites is between 3×10^5 and 5×10^4 (Crouch & Klee, 1980); for TnC, K_a is 2×10^7 for the high-affinity sites and 5×10^5 for the low-affinity sites (Potter & Gergely, 1975). Alternative interpretation is that the Ca^{2+} binding to the domains III and IV influences the domains I and II. At present, there is no evidence to prove this.

In the course of Ca^{2+} titration of F12, a single peak is observed at an average position of the two chemical shifts of Ca^{2+}-free and Ca^{2}-bound F12, and the peak position changes with varying content of Ca^{2+}. This means that the conformational change between the Ca^{2+}-free and -bound states in F12 with low-affinity sites for Ca^{2+} occurs at a rate more than 300 s^{-1} (the conditions of the fast-exchange process on the NMR time scale). F1 and F4 fragments also exhibit the fast transformation between the two states. In contrast to F12, F1, and F4, F34 shows two separate peaks at postions of resonances in Ca^{2+}-free and -bound states; peak intensities of the two change with varying content of Ca^{2+}. This indicates that the conformational change between the two states in F34 containing high-affinity sites for Ca^{2+} occurs at a rate less than 50 s^{-1} (the conditions of the slow-exchange process on the NMR time scale). Line broadening is observed upon the addition of Ca^{2+} in all fragments. This suggests that exchange processes are in the fast intermediate region on the NMR time scale for F12, F1, and F4 and in the slow intermediate region for F34 (Kimura, 1983). This is in agreement with the recent results of a Cd^{113} NMR study on proteolytic fragments of CaM (Andersson et al., 1983). The high- and low-affinity Ca^{2+}-binding domains of TnC exhibit similar exchange behaviors to those of CaM (Levine et al., 1977; Hincke et al., 1981). The other Ca^{2+} binding proteins that contain two Ca^{2+}-binding domains, parvalbumin (Birdsall et al., 1979) and porcine intestinal protein (Shelling et al., 1983), show spectra only in the slow exchange process. This is because these proteins are composed of only the two high-affinity domains.

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