Nuclear Magnetic Resonance Studies on Calmodulin: Spectral Assignments in the Calcium-Free State†

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ABSTRACT: The 400-MHz proton magnetic resonance spectra of calcium-free scallop testis calmodulin (CaM) and pig brain CaM were observed. Detailed spectral assignments were made by resolution enhancement, spin decoupling, and nuclear Overhauser enhancement (NOE) experiments as well as pH titration. Comparison between spectra of scallop testis CaM and pig brain CaM were also utilized for the assignment. Previous assignments for tyrosine-99, histidine-107, ϵ-trimethyllysine-115, and tyrosine-138 [Seamon, K. B. (1980) *Biochemistry 19*, 207; Krebs, J., & Carafoli, E. (1982) *Eur. J. Biochem. 124*, 619] were confirmed. Phenylalanine-99 and threonine-143 of scallop testis CaM were identified. Sixteen methyl resonances from one isoleucine, two valines, nine methionines, and the amino-terminal acetyl group were identified. First-stage assignments were made of resonances arising from seven phenylalanines. The uniquely high field shifted phenylalanine resonance previously reported by Seamon was found to consist of two doublets from the two pairs of δ protons of two phenylalanines. The NOE experiments showed that the two phenylalanines are located closely to each other. The large high-field shifts of these phenylalanines were accounted for the ring-current effects due to their proximity. An isoleucine and a valine of which methyl resonances appear at high fields were found to be situated closely to each other. It was found that two δ protons and two ϵ protons of almost all aromatic residues are magnetically equivalent, suggesting that the local structure of aromatic residues is so flexible as to permit the rapid flipping motion of the ring.

Calmodulin (CaM)[1] was first discovered as a Ca^{2+}-dependent activator of cyclic nucleotide phosphodiesterase in rat brain by Kakiuchi et al. (1970). Cheung (1970) also discovered independently the same protein from bovine brain. The CaM–Ca^{2+} complex has been known to show multifunctional properties in cellular regulation (Cheung, 1980). CaM is widely distributed in vertebrates, in invertebrates, and also in plants (Klee et al., 1980), suggesting that it is ubiquitous in eukaryotes. It is a relatively small (M_r 16 700), heat- and acid-stable protein. The amino acid sequence has been determined for calmodulins (CaM's) isolated from various sources including rat testis (Dedman et al., 1978), bovine uterus (Grand & Perry, 1978), bovine brain (Watterson et al., 1980), sea anemone muscle (Takagi et al., 1980), *Tetrahymena pyriformis* (Yazawa et al., 1981), and scallop adductor muscle (Toda et al., 1981). The results obtained so far show that at most only 8% of amino acid residues vary throughout evolution, suggesting that CaM is quite conservative. For the most part, CaM's lack cysteine and tryptophan and contain one unusual amino acid, ϵ-trimethyllysine.

There have been a number of physicochemical studies on CaM (Kuo & Coffee, 1976; Liu & Cheung, 1976; Wolff et al., 1977; Klee, 1977; Dedman et al., 1977; Walsh & Stevens, 1977; Richman & Klee, 1978; Crouch & Klee, 1980; Seamon, 1980; Seamon & Moore, 1980; Krebs & Carafoli, 1982). These show that 1 mol of CaM binds up to 4 mol of Ca^{2+} and that the conformation changes as a consequence of binding of Ca^{2+}. Among these, ^1H NMR studies (Seamon, 1980; Krebs & Carafoli, 1982) indicate that the conformational change occurs in two steps, in a manner similar to that of troponin C (Levine et al., 1977; Hincke et al., 1981).

NMR spectroscopy is quite useful for the study of subtle changes in the microenvironment of specific residues if resonances can be assigned to specific residues. Although Seamon and Krebs et al. reported assignments of resonances of Tyr-99, His-107, Tml-115, and Tyr-138 in bovine brain CaM, additional spectral assignments are required to study the conformation of CaM in more detail. It has also been reported that some resonances in aromatic and methyl regions are shifted upfield due to the ring-current effect and that these resonances are very sensitive to the Ca^{2+}-induced conformational change. However, these resonances have not been assigned yet.

The purpose of this work is to make more detailed spectral assignments that are prerequisite for the conformational study (Ikura et al., 1983). Spin-decoupling experiments and NOE difference spectroscopy experiments were carried out for the spectral assignments. We also performed a comparative study on spectra between scallop testis and pig brain CaM's. Resonances of some substituted residues in the two proteins could be identified. The spectral assignments of CaM in the Ca^{2+}-free state are summarized.

Experimental Procedures

Preparation of Protein Samples. Scallop testis and pig brain CaM's were isolated and purified as described previously (Yazawa et al., 1980). The purity was confirmed by NaDodSO$_4$ gel electrophoresis and ultraviolet absorption spectra. The amount of residual Ca^{2+} in the protein prepared by the TCA precipitation was less than 0.03 mol/mol of the protein, as measured by a Hitachi 208 atomic absorption spectrometer. All glassware used for NMR measurements of Ca^{2+}-free CaM was washed with a 10 mM EGTA (pH ~10) solution and was rinsed by deionized and distilled water. Protein samples were lyophilized in D_2O at least 3 times to replace exchangeable protons with deuteriums. Solutions of 1.5 mM CaM containing 0.2 M KCl in D_2O were usually used for NMR measurements.

The pH titration was performed by adding 4% KOD or 4% DCl solutions to the samples. pH values quoted here are direct meter readings measured on a Horiba F7CL pH meter and

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[1] Abbreviations: CaM, calmodulin; Tml, ϵ-trimethyllysine; NOE, nuclear Overhauser enhancement; NaDodSO$_4$, sodium dodecyl sulfate; TCA, trichloroacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TSP, (trimethylsilyl)propionic acid; NMR, nuclear magnetic resonance.

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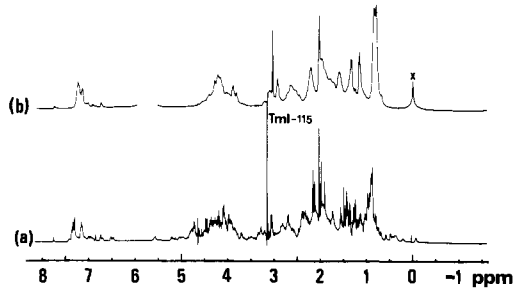

FIGURE 1: (a) 400-MHz ^{1}H NMR imaginary difference spectrum of Ca^{2+}-free scallop testis calmodulin at pH 8.8 and at 40 °C; (b) spectrum of Ca^{2+}-free scallop testis calmodulin in 6 M urea at pH 8.5 and at 23 °C. [Calmodulin] = 1.5 mM; [KCl] = 0.2 M. Peak X at about 0.1 ppm in spectrum b is due to unknown impurity. Resonance assigned to Tml-115 is labeled.

are not corrected for the deuterium isotope effect.

NMR Measurement. ^{1}H NMR measurement were performed at a frequency of 400 MHz on a JEOL FX-400 spectrometer operating in a pulse Fourier-transform mode with quadrature detection. All spectra were obtained from accumulation of 512–1024 free-induction decays after each 45° pulse (5 μs) repeated every 4 s and observed over a range of 4000 Hz, corresponding to 16K data points. Digital resolution was 0.5 Hz with digital filtering of 0.2 Hz. Chemical shifts were measured in parts per million (ppm) from an internal standard of TSP. The residual HDO peak was suppressed by the gated decoupling method (Hoult & Richards, 1975).

(I) Resolution Enhancement. To enhance apparent resolution in the spectra, two kinds of procedures were employed. One is imaginary difference spectrum. The imaginary part of a spectrum is shifted downfield by four points (about 2 Hz) and subtracted from the original imaginary part. The resulting spectrum corresponds to the derivative of the imaginary part in which apparent resolution is markedly enhanced. To conserve the integrated intensity, the difference spectrum is doubled in intensity and added to the original real spectrum. The resulting spectrum is referred to as the imaginary difference spectrum (S. Takahashi, personal communication). The other is smoothing difference spectrum. The real part of a spectrum is smoothed by performing five-point averages and subtracted from the original real part. The resulting spectrum is referred to as the smoothing difference spectrum.

(II) Spin-Decoupling Experiment. Spin-decoupling experiments were performed by time-shared irradiation at a given resonance peak. During these double-resonance experiments, the residual HDO peak was decoupled in a gated manner with another decoupler unit.

(III) Nuclear Overhauser Enhancement Difference Spectroscopy. NOE difference spectroscopy was performed as follows. A weak presaturation pulse of a frequency f_A that saturates selectively a resonance peak at f_A is applied prior to the observed pulse, and subsequently, the free-induction decay is acquired. After a given number of accumulations (typically eight scans), data are stored. Then, the frequency of the presaturation pulse is switched to f_B; data are accumulated in the same way as before and stored. The difference between the two data sets is calculated. The entire procedure is repeated more than 500 times, and the difference is accumulated. The frequency of f_A was set at a resonance of interest, and f_B was set in the off-resonance region of the spectrum, usually at 10 ppm upfield from TSP.

Results

Assignments of Ca^{2+}-Free Scallop Testis Calmodulin. Figure 1a shows an imaginary difference spectrum of Ca^{2+}-free scallop testis CaM at pH 8.8 and at 40 °C. The most intense singlet line at 3.13 ppm is assigned to ϵ-trimethyl protons of Tml-115 as assigned previously by Seamon (1980). Singlet resonances observed in the vicinity of 2 ppm are due to methyl protons of nine Met residues and the N-terminal acetyl group. The verification of singlets for these methyl resonances was performed by observing spin-echo spectra, in which singlet lines have the normal phase whereas doublet lines and two outer components of triplet lines are in the inverted phase (Campbell et al., 1975a). They are designated as S1–S10 and indicated in Table I and Figure 3. Two resonances of S4 and S5 overlap each other. On the other hand, in a spectrum of the protein denatured by 6 M urea (Figure 1b), a strong singlet line due to methyl groups of all Met's appears at 2.1 ppm. These results indicate that microenvironments around Met groups differ from residue to residue in the native protein, whereas the microenvironments of all Met residues in the denatured protein are similar and these residues are probably exposed to water.

Table I: Assignments of Singlet Methyl Resonances from Nine Methionines, N-Terminal Acetyl Group, and ϵ-Trimethyllysine of Scallop Testis Calmodulin

designation	chemical shift[a]	designation	chemical shift[a]
S1	2.14	S7	1.97
S2	2.13	S8	1.95
S3	2.10	S9	1.87
S4	2.00	S10	1.47
S5	2.00	Tml-115, ϵ-trimethyl	3.13
S6	1.99		

[a] ppm from TSP. Measurements were made at 40 °C.

Figure 2a shows the low-field region of a smoothing difference spectrum of CaM at pH 8.8 and at 40 °C. The low-field region consists of resonances from ring groups of nine Phe, one Tyr, and one His. In the native spectrum, a large number of resonances shift from normal positions at which the resonances are observed in the denatured state (Figure 1b). Most marked upfield shifts may be attributable to the ring-current effect of aromatic residues (for example, peaks A and B in Figure 2a).

Experiments of pH titration were performed in order to identify resonances of Tyr-138 and His-107. A doublet peak that was observed at 6.67 ppm at pH 8.8 shifts upfield significantly as the pH increases above 10 in parallel with a relatively small upfield shift of a doublet peak at 6.73 ppm. Thus, we assigned the two doublet resonances at 6.37 and 6.67 ppm to two equivalent δ protons and two equivalent ϵ protons of Tyr-138, respectively.

A singlet peak that was observed at 7.73 ppm at pH 8.8 largely shifts downfield as pH decreases below 8 in parallel with a relatively small downfield shift of a singlet peak at 6.84 ppm. Hence, we assigned the two singlet resonances at 7.73 and 6.84 ppm to H2 and H4 protons of His-107, respectively. These assignments of Tyr-138 and His-107 are in agreement with the previous results of bovine brain CaM (Seamon, 1980).

All other resonances in the aromatic region are due to nine Phe residues. In order to assign these Phe resonances, extensive spin-decoupling experiments were carried out. Figure 2b shows two typical spin-decoupling spectra with time-shared irradiation of two triplets at 6.90 (A′) and 7.15 ppm (B′), respectively. Irradiation at 6.90 ppm (A′) changes a doublet peak at 6.50 ppm (A) and a triplet peak at 7.42 ppm (A″) into singlet lines. Similarly, irradiation at 7.15 ppm (B′) makes changes in a doublet at 6.46 ppm (B) and a triplet at 7.39 ppm (B″). Thus, we assigned resonances A, A′, and A″ respectively to two equivalent δ protons, two equivalent ϵ protons, and the

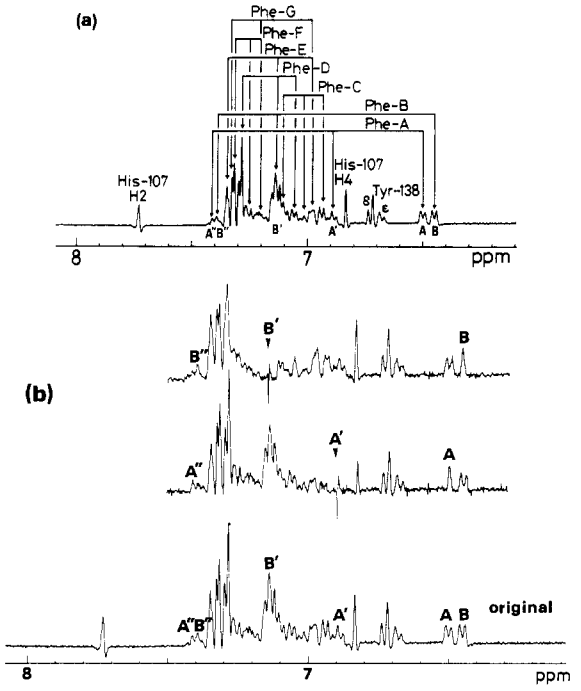

FIGURE 2: (a) Aromatic region of resolution-enhanced smoothing difference spectrum of Ca^{2+}-free scallop testis calmodulin at pH 8.8 and at 4 °C: [calmodulin] = 1.5 mM; [KCl] = 0.2 M. Resonances assigned to Tyr-138, His-107, and seven phenylalanines are labeled. Peak symbols are marked under the spectrum. (b) Aromatic regions of spin-decoupling spectra of uniquely high field shifted phenylalanine resonances. Spectra are resolution-enhanced by the smoothing difference method: (bottom) control spectrum with no irradiation; (middle) irradiation at 6.90 ppm (peak A'); (top) irradiation at 7.15 ppm (peak B'). Peaks irradiated are indicated by arrows. Decoupling effects observed are marked with the same symbols as shown in (a).

Table II: Assignments of Aromatic Resonances of Scallop Testis Calmodulin

residue	chemical shift[a] δ	ε	ζ
His-107	7.73 (s) (H2)[b]	6.84 (s) (H4)	
Tyr-138	6.73 (d)	6.67 (d)	
Phe-A	6.50 (d)	6.90 (t)	7.42 (t)
Phe-B	6.46 (d)	7.15 (t)	7.39 (t)
Phe-C	6.94 (d)	7.11 (t)	7.02 (t)
Phe-D	7.06 (d)	7.14 (t)	7.29 (t)
Phe-E (Phe-99)	6.98 (d)	7.15 (t)	7.34 (t)
Phe-F	7.26 (d)	7.32 (t)	7.2 (t)
Phe-G	7.34 (d)	7.21 (t)	6.99 (t)
Tyr-99[c]	6.82 (d)	7.19 (d)	

[a] ppm from TSP. Measurements were made at 40 °C and at pH 8.8. [b] Multiplicity. [c] This was obtained for pig brain calmodulin.

ζ proton of one Phe residue and, similarly, B, B′, and B″ to corresponding protons of another Phe residue. Performing further decoupling experiments for other Phe resonances, we identified resonances of seven Phe residues out of nine; these are denoted as Phe-A, -B, -C, -D, -E, -F, and -G (Figure 2a). Resonances of two remaining Phe residues have not yet been assigned. These may be buried in a crowded spectral region between 7.1 and 7.35 ppm. The chemical shift data for the aromatic resonances are summarized in Table II.

Figure 3 shows the high-field region of a smoothing difference spectrum of CaM at pH 8.8 and at 40 °C. Scallop testis CaM contains 71 methyl groups of nine Leu, eight Ile, seven Val, ten Ala, and thirteen Thr residues (Toda et al., 1981). In the denatured state (Figure 1b), Leu, Ile, and Val exhibit strong methyl resonances with a maximum at 0.9 ppm

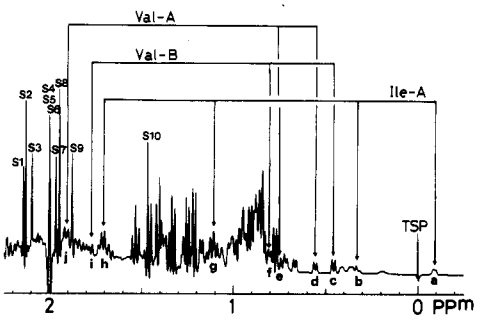

FIGURE 3: Aliphatic region of resolution-enhanced smoothing difference spectrum of Ca^{2+}-free scallop testis calmodulin at pH 8.8 and at 40 °C: [calmodulin] = 1.5 mM; [KCl] = 0.2 M. Resonances assigned to two valines and one isoleucine are labeled. Peak symbols are marked under the spectrum.

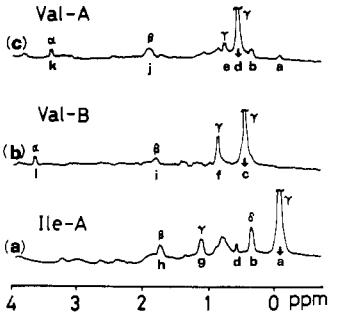

FIGURE 4: Aliphatic regions of NOE difference spectra of Ca^{2+}-free scallop testis calmodulin at pH 8.5 and at 40 °C: [calmodulin] = 1.8 mM; [KCl] = 0.2 M; irradiation pulse duration 1.8 s. Negative NOE's are phased so as to give positive peaks. (a) Irradiation of peak a (Ile-A γ-methyl); (b) irradiation of peak c (Val-B, γ-methyl); (c) irradiation of peak d (Val-A γ-methyl). Assignments are indicated. Peak symbols correspond to those in Figure 2.

while Ala and Thr residues show resonances at 1.2 ppm. Decreases in intensity around 0.9 ppm and also around 1.2 ppm in the native spectrum (Figure 1a) are accounted for mostly by the upfield ring-current shift from the unperturbed position to the region of 0.8 to −0.1 ppm.

In order to assign these ring current shifted methyl peaks, we performed NOE difference experiments as well as spin-decoupling experiments. Figure 4 shows three NOE difference spectra in which three different doublet methyl peaks were irradiated. In spectrum a in which a doublet methyl peak, a, appearing at the highest field is irradiated, the NOE effect is observed to a relatively great extent on a triplet methyl peak, b. Since only Ile shows both doublet and triplet methyl resonances, peak a and peak b are assigned to Ile. This assignment of peak a is at variance with that reported on bovine brain CaM recently by Krebs & Carafoli (1982), who stated that this peak is due to either Leu, Val, Ala, or Thr.

Spin-decoupling experiments as well as spin-echo experiments were also carried out to confirm the assignments. Time-shared irradiation of peak h at 1.72 ppm sharpens the doublet methyl peak a at −0.09 ppm. Time-shared irradiation of peak g at 1.10 ppm changes the triplet methyl peak b at 0.33 ppm into a doublet. Thus, we assigned peaks a, b, g, and h to γ-CH_3, δ-CH_3, γ-CH, and β-CH of one of eight Ile residues, referred to as Ile-A.

In the NOE difference spectrum b in which a doublet methyl peak, c, was irradiated, doublet peak f in the methyl region and doublet peak k in the α-methine region could be observed. Spin-decoupling experiments were made on these peaks. Time-shared irradiation of peak i in the β-methine region changes not only the doublet methyl resonance c but also another doublet peak, f, into singlet peaks.

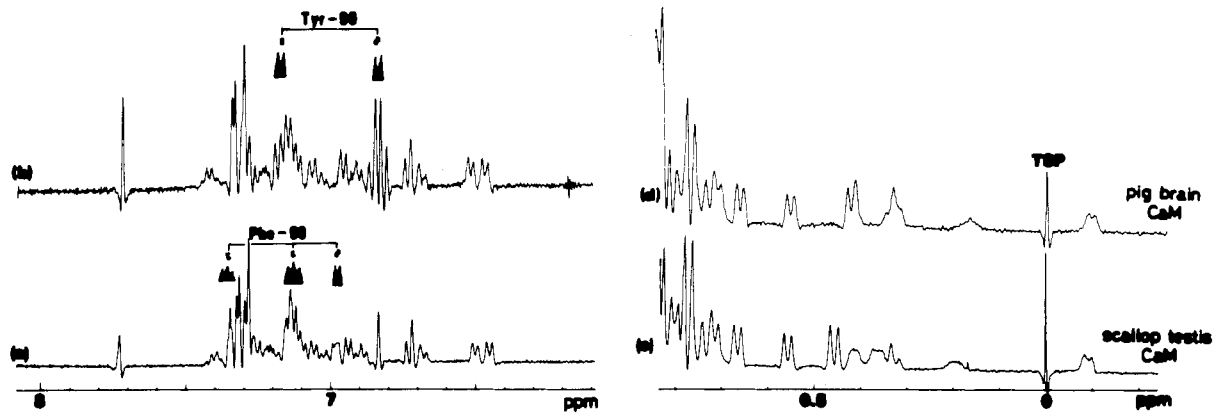

FIGURE 5: Comparison between scallop testis and pig brain calmodulins at pH 8.8 and at 40 °C: [calmodulin] = 1.5 mM; [KCl] = 0.2 M. Spectra are resolution-enhanced by the smoothing difference method. (a and b) High-field aliphatic spectral region; (c and d) low-field aromatic spectral region. Resonances assigned to Phe-99 of scallop testis calmodulin and to Tyr-99 of pig brain calmodulin are labeled.

Table III: Assignment of Aliphatic Resonances of Scallop Testis Calmodulin

residue	chemical shift[a] α	β	γ	δ
Ile-A	*b*	1.72[c]	1.10 (CH) −0.09 (d) (CH_3)	0.33 (t)
Val-A	3.39 (d)	1.91	0.75 (d) 0.55 (d)	
Val-B	3.36 (d)	1.78	0.85 (d) 0.46 (d)	
Thr-143			1.21 (d)	

[a] ppm from TSP. Measurements were made at 40 °C. [b] Not determined. [c] Multiplicity.

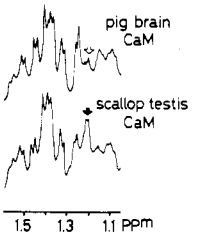

FIGURE 6: Evidence for finding of Thr-143 γ-methyl resonance of scallop testis calmodulin. Difference is marked by black and white arrows. Experimental conditions are same as those in Figure 5. Spectra are not resolution enhanced.

In the NOE difference spectrum c in which a doublet methyl peak, d, was irradiated, doublet peaks a and e in the methyl region and doublet peak f in the α-methine region were observed. In the spin-decoupling experiments, time-shared irradiation of peak j in the β-methine region changes the two doublet methyl resonances e and d into singlet peaks simultaneously.

Among all amino acids contained in CaM, only Val and Leu exhibit two doublet methyl resonances, and only Val and Ile show a doublet α-methine resonance. Thus, we assigned peaks d, e, j, and k, respectively, to γ-CH_3, γ-CH_3, β-CH, and α-CH of one of seven Val residues and peaks c, f, i, and l to corresponding protons of another Val residue. These Val residues are referred to as Val-B and Val-A. The assignments for Ile-A, Val-A, and Val-B are indicated in Figure 3, and the chemical shift data are summarized in Table III. Krebs & Carafoli (1982) reported that a resonance appearing at 0.12 ppm for bovine brain CaM at pH 7.5 and at 30 °C is a triplet and that the peak must be due to an Ile residue. We also observed a resonance in this region, but we could not confirm their assignment, since the peak is too broad to determine the multiplicity by the spin-echo experiment.

Spectral Comparison between Scallop Testis and Pig Brain Calmodulins. ^{1}H NMR spectra of pig brain CaM were found to be identical with those of bovine brain CaM, which have been reported previously (Seamon, 1980; Krebs & Carafoli, 1982). Phe-99, Thr-143, and Ser-147 in scallop testis CaM are replaced, respectively, by Tyr-99, Gln-143, and Ala-147 in pig brain CaM. Spectra of the two CaM's are compared as shown in Figure 5. Some differences are found in the aromatic region. It is likely that such differences are largely due to the replacement of Tyr-99 in pig brain CaM with Phe-99 in scallop testis CaM. Thus, we identified resonances of Tyr-99 of pig brain CaM and also those of Phe-99 of scallop testis CaM. Phe-E assigned previously turns out to be Phe-99. The assignment of Tyr-99 of pig brain CaM was confirmed by spin-decoupling experiments.

In the high-field region where ring current shifted methyl peaks appear, some differences are also noticeable in the vicinity of 0.4 ppm. At least, it is certain that peaks at about 0.4 ppm in scallop testis CaM are missing in pig brain CaM. The orientation of the phenyl ring(s) of the aromatic residue(s) relative to the methyl group(s) of scallop testis CaM is probably different from that of pig brain CaM.

A doublet methyl peak observed at 1.21 ppm of scallop testis CaM is also missing in pig brain CaM (Figure 6). If we take into account only the difference in amino acid sequence between two CaM's, this peak can be assigned to γ-methyl protons of Thr-143, the chemical shift of which is close to that observed in the denatured state.

Discussion

Tyr-99 of bovine brain CaM is replaced by Phe-99 in scallop testis CaM and also in octopus CaM, NMR spectra of which have been reported previously by Seamon & Moore (1980). The aromatic portions of NMR spectra of scallop testis CaM and octopus CaM are very similar to each other, whereas the two spectra are different from that of bovine brain CaM. The complete amino acid sequence of octopus CaM has not been reported yet. The results obtained here suggest that the amino acid sequence of octopus CaM is the same as that of scallop testis CaM at least with respect to aromatic residues.

The spectra shown in Figure 1 indicate the existence of a definite tertiary structure of Ca^{2+}-free CaM in the presence of 0.2 M KCl. In this study, all spectra were run under the conditions of 0.2 M KCl. We confirmed that the spectrum of Ca^{2+}-free CaM in 0.2 M KCl is very similar to that observed under the conditions without any salt (M. Ikura et al., unpublished results) and quite different from that in the

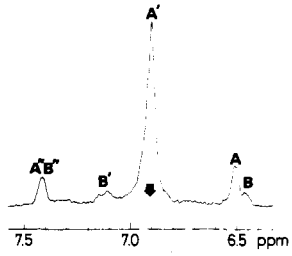

FIGURE 7: Aromatic region of NOE difference spectrum of Ca^{2+}-free scallop testis calmodulin at pH 8.5 and at 40 °C with irradiation of peak A′ (Phe-Aε): [calmodulin] = 1.8 mM; [KCl] = 0.2 M; irradiation pulse duration 0.5 s. Negative NOE's are phased so as to give positive peaks. Peak symbols correspond to those in Figure 2.

Ca^{2+}-saturated state. Effects of Ca^{2+} will be discussed in a subsequent paper (Ikura et al., 1983).

Seamon (1980) assigned a resonance observed at 6.49 ppm for Ca^{2+}-free bovine brain CaM at pH 7.5 and 23 °C to a uniquely high field shifted Phe residue. In the present study, we found that the resonance consists of two doublets, which are assigned to δ protons of two Phe residues, Phe-A and Phe-B.

Figure 7 shows an NOE difference spectrum in which the resonance A′ due to ε protons of Phe-A is irradiated. It is found that NOE effects are observable not only for resonances A and A″ of the same Phe-A but also for resonances B, B′, and B″ of another Phe-B. These results indicate that Phe-A and Phe-B are located closely to each other. It is quite probable that large high-field shifts of the two Phe δ resonances are caused by the ring-current effect due to the proximity to each other. Chemical shift positions of Phe-A and Phe-B resonances of scallop testis CaM are identical with those of pig brain CaM (Figure 5), bovine brain CaM (Seamon, 1980), and octopus CaM (Seamon & Moore, 1980), suggesting that Phe-A and Phe-B in all CaM's studied are assembled in a similar manner.

When the γ-methyl resonance of Val-A is irradiated (Figure 4c), NOE effects are observed on two methyl resonances of Ile-A (peaks a and b). Furthermore, when the γ-methyl resonance of Ile-A is irradiated (Figure 4a), the NOE effect is observed on the γ-methyl resonance of Val-A (peak d). These results indicate that Ile-A and Val-A are also located closely to each other.

Our assignments for the ring resonances of Tyr-99 of pig brain CaM, which were made by the comparison with scallop testis CaM, are in agreement with those of bovine brain CaM reported by Krebs & Carafoli (1982) but not those by Seamon (1980). The resonance of Tyr-138 of all CaM's studied appears at relatively high fields as compared with Tyr-99 of pig brain and bovine brain CaM's. The p*K* value of Tyr-138 estimated by a pH titration of the Tyr-138 resonance was 12.0, in contrast with the p*K* value of Tyr-99, 10.4 (Richman & Klee, 1978). These results indicate that the Tyr-138 residue of all CaM's is situated in a more hydrophobic environment as compared to Tyr-99, which seems to be largely exposed to water.

It has been reported that two δ protons and also two ε protons of some Tyr and Phe residues in several proteins are not magnetically equivalent (Campbell et al., 1975b): Phe-46 and Tyr-48 in horse ferrocytochrome *c* (Cookson et al., 1978); Tyr-23, Tyr-35, and some Phe's in basic pancreatic trypsin inhibitor (Wagner et al., 1976). The present results indicate that two δ protons and two ε protons of Tyr-99, Tyr-138, and most Phe residues in CaM are magnetically equivalent. Such equivalence can result from the rapid flipping motion of the ring about the C_β–C_γ bond. It is suggested that the local structure in the vicinity of aromatic residues of CaM is so flexible as to permit the rapid flipping motion of the ring.

Registry No. L-Tyrosine, 60-18-4; L-histidine, 71-00-1; ε-trimethyllysine, 19253-88-4; L-phenylalanine, 63-91-2; L-threonine, 72-19-5; L-isoleucine, 73-32-5; L-valine, 72-18-4; L-methionine, 63-68-3; L-leucine, 61-90-5.

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