Nuclear Magnetic Resonance Studies on Calmodulin: Calcium-Induced Conformational Change†

Mitsuhiko Ikura, Toshifumi Hiraoki, Kunio Hikichi,* Toshiaki Mikuni, Michio Yazawa, and Koichi Yagi

ABSTRACT: The 400-MHz ^1H nuclear magnetic resonance (NMR) studies were carried out on the Ca^{2+}-induced conformational change of calmodulins (CaM's) isolated from scallop testis and pig brain. The resonances were found to be classified approximately into three groups. The resonances of group I, which are perturbed by the binding of Ca^{2+} to the high-affinity sites, include those of tyrosine-138, ϵ-trimethyllysine-115, histidine-107, tyrosine-99, etc. The previous assignments for tyrosine- (Tyr) 138 [Seamon, K. B. (1980) *Biochemistry 19*, 207] were corrected. The resonances of group II, which are affected by the binding of Ca^{2+} to the low-affinity sites, include those of a phenylalanine (Phe), a high field shifted methyl, and a low field shifted α-methine. Group III (related to the binding of Ca^{2+} to both the high- and low-affinity sites) includes the resonances of a Phe, a high field shifted methyl, and threonine-143. It is concluded that sites III and IV are the high-affinity sites. The off-rate of Ca^{2+} from the high-affinity sites is slower than 50 s^{-1} while the off-rate from the low-affinity sites is faster than 600 s^{-1}. In the Ca^{2+}-free state, there exists a hydrophobic region containing three phenylalanine (probably Phe-89, Phe-92, and Phe-141), a valine, and an isoleucine in the vicinity of sites III and IV. Tyr-138 is distant from these amino acids. Upon binding of Ca^{2+} to the high-affinity sites, one of the Phe residues and the valine approach Tyr-138. Similar structural changes were observed between CaM and troponin C when Ca^{2+} ions are bound to the high-affinity sites. CaM changes in a somewhat different way from troponin C when Ca^{2+} ions are bound to the low-affinity sites.

Calmodulin (CaM)[1] is a ubiquitous Ca^{2+}-binding protein and plays a pivotal role in cellular regulation (Cheung, 1980). The amino acid sequence of CaM is homologous to those of other Ca^{2+}-binding proteins: TnC and parvalbumin. These proteins function in a different manner [for reviews, see Kretsinger (1980a) and Klee et al. (1980)]. It is of particular importance to clarify the mechanism of the Ca^{2+} binding to CaM in order to understand the Ca^{2+}-dependent activation and regulation of enzymes. There have been a number of studies on the Ca^{2+}-binding properties of CaM (Teo et al., 1973; Wolff et al., 1977; Crouch & Klee, 1980); these indicate that 1 mol of CaM binds 4 mol of Ca^{2+}. Kretsinger (1980b) proposed a predicted conformation for CaM, which was based on the crystal structure of parvalbumin (Moews & Kretsinger, 1975). There are four Ca^{2+}-binding sites, each of which may have the same conformation as does the EF hand of parvalbumin. These four binding sites are labeled as sites I, II, III, and IV from the N to the C terminal.

Controversy has arisen recently over the scheme of the Ca^{2+} binding, in particular with respect to the location of the high- and low-affinity sites. From the results of Tb^{3+} luminescence spectroscopic studies, Kilhoffer et al. (1980a,b) and Wang et al. (1982) concluded that sites I and II are the high-affinity sites. Although the same technique was used, Wallace et al. (1982) reached a conclusion that either of sites I or II is the highest affinity site and that either of sites III or IV is the next highest affinity site. On the other hand, from the NMR results, Seamon (1980) judged site III to have the highest affinity. Our ESR studies on spin-labeled CaM suggested that sites III and IV are the high-affinity sites (Yagi et al., 1982). Hincke et al. (1981b) carried out laser photo-CIDNP ^1H NMR experiments and reported that the high-affinity site is site IV. On the contrary, a recent ^1H NMR study on bovine brain CaM indicated that site IV is the lowest affinity site (Krebs & Carafoli, 1982).

Upon binding of Ca^{2+}, CaM undergoes a large conformational change. Previous ^1H NMR study (Seamon, 1980) showed that the conformational change occurs in two steps with increasing Ca^{2+} content. It is, therefore, of considerable importance to know the conformation of CaM at various Ca^{2+} contents in order to understand CaM regulation of enzymes, since more than 2 mol of Ca^{2+}/mol of CaM is required for the activity.

Tyr-138 of CaM is located in site IV and has been employed as a useful probe for studying the properties of the binding site. Seamon (1980) indicated from his ^1H NMR assignments that resonances of Tyr-138 change in a whole range of Ca^{2+} contents from 0 to 4 mol/mol of CaM. This behavior is quite different from that of Tyr-150 of cardiac TnC (Hincke et al., 1981a), although the latter occupies a position homologous to the former. The resonances of Tyr-150 vary only when the first 2 mol of Ca^{2+} is added, and no change can be observed upon addition of Ca^{2+} in excess of 2 mol/mol of CaM. It has been also reported that in addition to the resonances of Tyr-138 several other resonances are very sensitive to Ca^{2+} binding. Valuable informations about structure and structural changes in CaM would be reflected by these resonances, most of which have not been, however, assigned to specific amino acids yet.

In a preceding paper (Ikura et al., 1983), we reported detailed spectral assignments of CaM in the Ca^{2+}-free state. In this paper, we will present the results of further studies on the kinds of CaM isolated from scallop testis and pig brain at various Ca^{2+} contents; Phe-99, Thr-143, and Ser-147 in the former are replaced respectively by Tyr-99, Gln-143, and Ala-147 in the latter. Comparison will be made between our results and those previously reported for bovine brain CaM and skeletal and cardiac TnC. Finally, we will present sug-

† From the Department of Chemistry (T.M., M.Y., and K.Y.), the Department of Polymer Science (T.H. and K.H.), and the High-Resolution NMR Laboratory (M.I.), Faculty of Science, Hokkaido University, Sapporo 060, Japan. *Received October 27, 1982.* This work was supported in part by the Ministry of Education, Science, and Culture of Japan and the Muscular Dystrophy Association of America, Inc.

[1] Abbreviations: CaM, calmodulin; TnC, troponin C; Tml, ϵ-trimethyllysine; NOE, nuclear Overhauser enhancement; TSP, (trimethylsilyl)propionic acid; NMR, nuclear magnetic resonance; ESR, electron spin resonance; CIDNP, chemically induced dynamic nuclear polarization.

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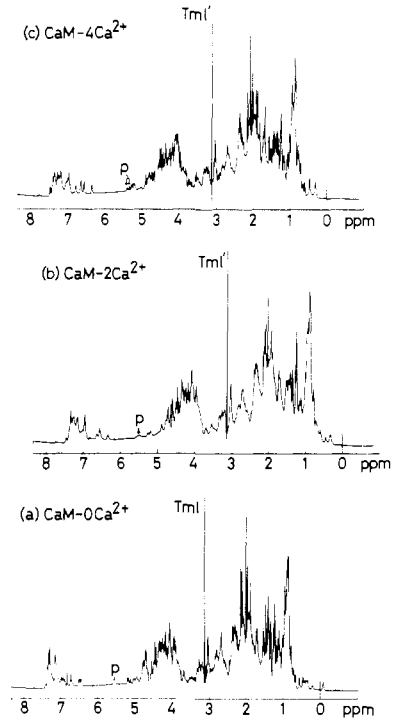

FIGURE 1: 400-MHz ^1H NMR imaginary difference spectra of scallop testis calmodulin at pH 8.8 and at 40 °C: [calmodulin] = 1.5 mM; [KCl] = 0.2 M. (a) $[Ca^{2+}]/[calmodulin] = 0.0$; (b) $[Ca^{2+}]/[calmodulin] = 2.0$; (c) $[Ca^{2+}]/[calmodulin] = 4.0$. Peaks Tml and Tml' correspond to the resonances of ϵ-trimethyl protons of Tml-115, and peak p corresponds to the low field shifted α resonance.

gested spatial arrangements of amino acids in the vicinity of Tyr-138 in the Ca^{2+}-free and -bound states.

Experimental Procedures

Preparation of Protein Samples. Scallop testis CaM and pig brain CaM were isolated and purified as described previously (Yazawa et al., 1980). Details of the preparation of samples for the NMR measurements were described in the preceding paper (Ikura et al., 1983). The Ca^{2+} titration was performed by addition of 20 mM or 100 mM $CaCl_2$ solution.

NMR Measurements. Experimental details of 400-MHz ^1H NMR measurements are the same as described in the preceding paper. D_2O solutions of 1.5 or 1.8 mM CaM containing 0.2 M KCl were used for the NMR measurements. All measurements were made at 23 or 40 °C. Chemical shifts are given in parts per million (ppm) from an internal standard of TSP.

Two kinds of resolution-enhanced spectra are shown; the imaginary difference spectrum and the smoothing difference spectrum. NOE difference spectra are also shown, in which the weak preirradiation pulse is applied for a period of 1.8 to ~2.0 s before the observing pulse. In the NOE difference spectra, the negative NOE effect was plotted as a positive peak.

Results

Ca^{2+}-Induced Spectral Changes of Scallop Testis Calmodulin. ^1H NMR spectra of scallop testis CaM were observed as a function of the molar ratio of Ca^{2+}/CaM. Spectra a, b, and c of Figure 1 show imaginary difference spectra of CaM at pH 8.8 and at 40 °C in the absence of Ca^{2+}, in the presence of 2 mol of Ca^{2+}/mol of CaM, and in the presence of 4 mol of Ca^{2+}/mol of CaM, respectively. It is clearly found that the spectra are greatly affected by the addition of Ca^{2+}. In particular, changes in spectra in the aromatic and high-field methyl regions are noticeable.

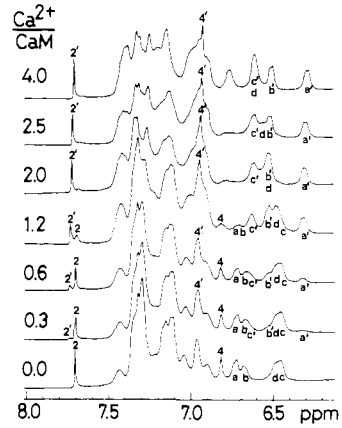

FIGURE 2: Aromatic regions of ^1H NMR spectra of scallop testis calmodulin as a function of molar ratio of Ca^{2+}/calmodulin at pH 8.8 and at 23 °C: [calmodulin] = 1.5 mM; [KCl] = 0.2 M. Peak assignments are as follows: (a and a') Tyr-138 δ; (b and b') Tyr-138 ϵ; (c and c') Phe-B δ; (d) Phe-A δ; (e) Phe-X δ; (2 and 2') His-107 H2; (4 and 4') His-107 H4.

The aromatic regions of expanded spectra of CaM at pH 8.8 and at 23 °C are shown in Figure 2 as a function of the molar ratio of Ca^{2+}/CaM. At molar ratios above 4, the spectra were essentially identical with those in the presence of 4 mol of Ca^{2+}/mol of CaM.

Assignments for H2 and H4 protons of His-107 in the Ca^{2+}-saturated state were made by a pH titration. We assigned two singlet peaks 2' and 4', respectively, to H2 and H4 protons of His-107 in the Ca^{2+}-saturated state. Chemical shifts of the resonances of H2 and H4 protons are plotted against the molar ratio of Ca^{2+}/CaM in Figure 3 and the intensities for H2 proton in Figure 4. The chemical shifts are found to be unchanged by the Ca^{2+} additions. On the other hand, it can be seen that peak 2 of His-107 H2 proton in the Ca^{2+}-free state progressively diminishes in intensity with increasing Ca^{2+} content and vanishes at molar ratios above 2. The resonance in the Ca^{2+}-saturated state shows a change complementary to that in the Ca^{2+}-free state. A similar change was also observed for the resonance of His-107 H4 proton: peak 4 in the Ca^{2+}-free state and peak 4' in the Ca^{2+}-saturated state.

The method of pH titration was also utilized for the assignment of Tyr-138 in the Ca^{2+}-saturated state. Two doublet peaks a' and b' indicated in Figure 2 were respectively assigned to two equivalent δ protons and two equivalent ϵ protons of Tyr-138. Confirmation of these assignments comes from spin-decoupling experiments.

In the intermediate range of Ca^{2+} content, the assignment of Tyr-138 is more difficult due to overlap of resonances. To overcome this difficulty, we observed NOE difference spectra, which are shown in Figure 5. In the Ca^{2+}-free state, irradiation at peak a assigned to δ protons of Tyr-138 produces peak b assigned to ϵ protons. At a molar ratio of 2, irradiation at peak a' gives an almost identical spectrum with that at the molar ratio of 4. Thus, we assigned peak a' to δ protons and peak b' to ϵ protons. These assignments were confirmed by spin-decoupling experiments. Figures 3 and 4 include the results for Tyr-138.

In the low-field spectral region shown in Figure 2, resonances except those of His-107 and Tyr-138 described above are all due to phenylalanine residues. Peaks c and d in the Ca^{2+}-free state were assigned to δ protons of one of the phenylalanines (Phe-B) and to those of another phenylalanine (Phe-A), respectively, in the preceding paper (Ikura et al., 1983). We carefully examined these two resonances as a function of Ca^{2+} content. Peak c (Phe-B) does not show any

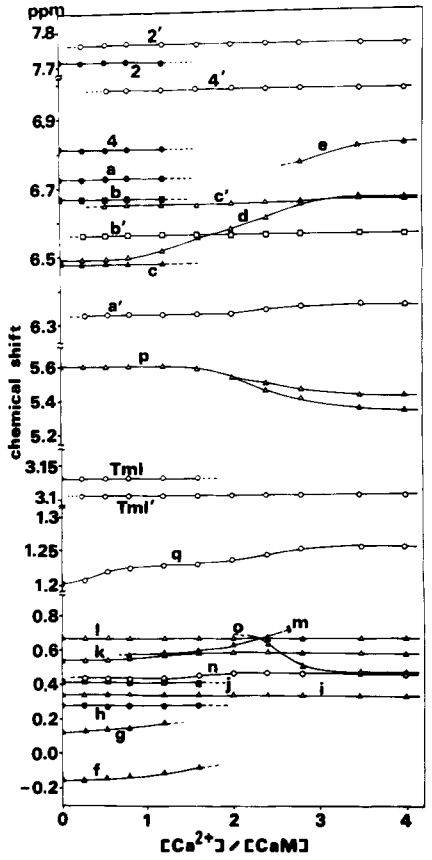

FIGURE 3: Chemical shift plot of some aromatic and aliphatic resonances as a function of molar ratio of Ca^{2+}/calmodulin. Experiments were performed as described in Figure 2. Peak symbols are the same as shown in Figures 1, 2, and 6. Peak q corresponds to the resonance of Thr-143 γ-methyl protons.

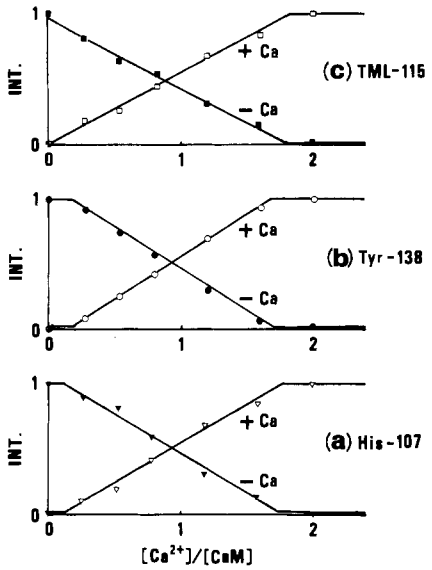

FIGURE 4: Relative intensities of typical resonances of scallop testis calmodulin as a function of molar ratio of Ca^{2+}/calmodulin. -Ca indicates the peak in the Ca^{2+}-free state; +Ca, the peak in the Ca^{2+}-bound state. (a) His-107 H2; (b) Tyr-138 δ; (c) Tml-115 ϵ-trimethyl.

change in chemical shift but shows a decrease in intensity and vanishes at molar ratio above 2. In company with the reduction in intensity of peak c, a new peak, c′, appears and grows in intensity, but no change in chemical shift is observed. Peak d (Phe-A) shifts downfield sigmoidally with increasing Ca^{2+} content. At molar ratios above 2, peak e appears and

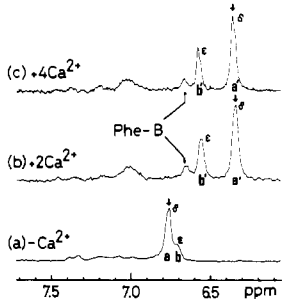

FIGURE 5: Aromatic regions of NOE difference spectra of scallop testis calmodulin at pH 8.5 and at 23 °C with irradiation of the resonance of Tyr-138 δ protons: [calmodulin] = 1.8 mM; [KCl] = 0.2 M. (a) $[Ca^{2+}]$/[calmodulin] = 0.0; (b) $[Ca^{2+}]$/[calmodulin] = 2.0; (c) $[Ca^{2+}]$/[calmodulin] = 4.0. The δ and ϵ resonances of Tyr-138 and the δ resonance of Phe-B are labeled.

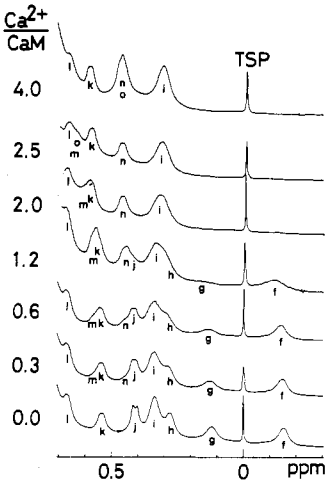

FIGURE 6: High-field methyl regions of ^{1}H NMR spectra of scallop testis calmodulin as a function of molar ratio of Ca^{2+}/calmodulin at pH 8.8 and at 23 °C. Experiments were performed as described in Figure 2. Peak assignments are as follows: (f) Ile-A γ-CH_3; (i) Ile-A δ-CH_3; (j) Val-B γ-CH_3; (k) Val-A γ-CH_3; peaks g, h, l, n, m, and o are not assigned.

shifts downfield as the third and fourth Ca^{2+} are added. This peak can be assigned to δ protons of one of the phenylalanine residues, labeled Phe-X. The crowded Phe region, 7.0–7.3 ppm, is largely perturbed upon addition of the first 2 mol of Ca^{2+}. Further perturbations are also observed as the molar ratio of Ca^{2+}/CaM is increased from 2 to 4.

Figure 6 shows high-field regions of expanded spectra at various molar ratios of Ca^{2+}/CaM at pH 8.8 and at 23 °C. Peak f assigned in the preceding paper to Ile-A γ-methyl and peak j assigned to γ-methyl of Val-B reduce in intensity with increasing Ca^{2+} content. Peaks g and h show a similar behavior as peaks f and j. In addition, peak m shifts downfield in parallel with an upfield shift of peak o in a range of Ca^{2+} content above 2. The addition of Ca^{2+} does not largely perturb peak i of Ile-A δ-methyl, peak k of Val-A γ-methyl, and peak l. Chemical shifts of those resonances are also plotted as a function of the molar ratio of Ca^{2+} to CaM in Figure 3.

The intense singlet lines at 3.11 ppm in the Ca^{2+}-saturated state and at 3.13 ppm in the Ca^{2+}-free state are assigned to ϵ-trimethyl protons of Tml-115 (Figure 1). As noted by Seamon (1980), with increasing molar ratio of Ca^{2+}/CaM from 0 to 2, Tml-115 shows two peaks. One peak at 3.11 ppm increases in intensity at the expense of another peak at 3.13 ppm. Chemical shifts and intensities for Tml-115 are also indicated in Figures 3 and 4, respectively.

Peak p at 5.60 ppm in the Ca^{2+}-free state (Figure 1a) can be assigned to α protons of unknown amino acids. This peak

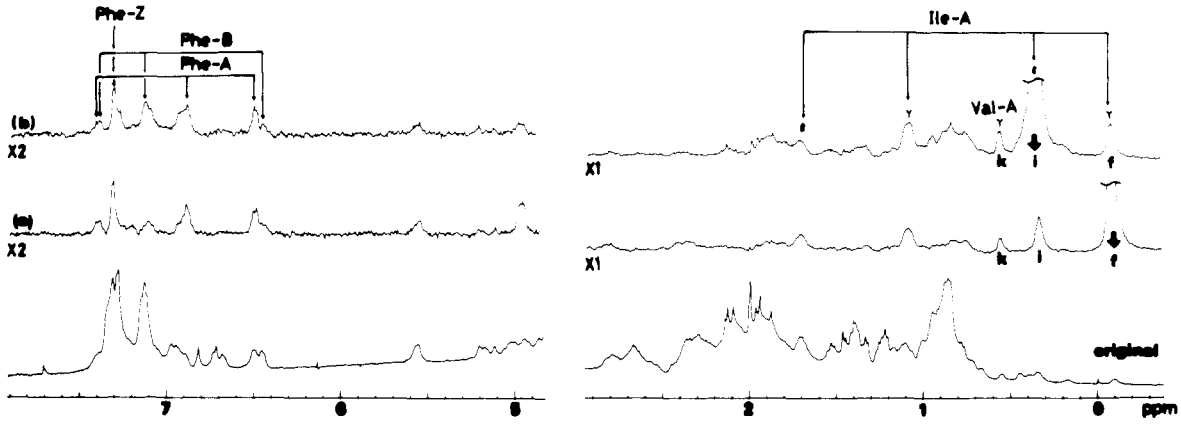

FIGURE 7: NOE difference spectra of Ca^{2+}-free scallop testis calmodulin at pH 8.5 and at 40 °C with irradiation of methyl resonances of Ile-A: [calmodulin] = 1.8 mM; [KCl] = 0.2 M. (a) Irradiation of Ile-A γ-methyl resonance (arrow); (b) irradiation of Ile-A γ-methyl resonance (arrow). NOE effects observed are marked. Peak symbols indicated under the spectrum correspond to those in Figure 6.

Table I: Chemical Shift Data[a] of Typical Resonances of Scallop Testis Calmodulin

	[Ca^{2+}]/[calmodulin]		
residue	0	2	4
His-107 H2	7.71	7.76	7.76
His-107 H4	6.81	6.98	6.98
Tyr-138 δ	6.73	6.33	6.35
Tyr-138 ϵ	6.67	6.57	6.57
Tml-115 ϵ-CH_3	3.13	3.11	3.11
Tyr-99[b] δ	6.82	6.79	6.79
Tyr-99 ϵ	7.19	6.95	6.95

[a] ppm from TSP. Measurements were made at pH 8.8 and at 23 °C. [b] This was obtained for pig brain calmodulin.

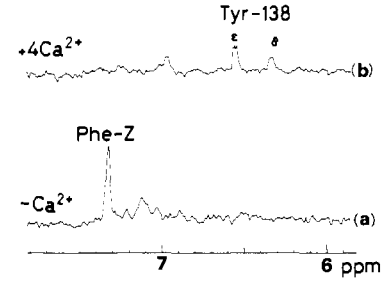

FIGURE 8: Aromatic regions of NOE difference spectra of scallop testis calmodulin at pH 8.5 and 40 °C with irradiation of γ-methyl resonance of Val-A: [calmodulin] = 1.8 mM; [KCl] = 0.2 M. (a) [Ca^{2+}]/[calmodulin] = 0.0; (b) [Ca^{2+}]/[calmodulin] = 4.0. NOE effects observed are marked.

appears at markedly low fields, presumably due to the ring-current effect. In the course of Ca^{2+} titration (Figure 4), no perturbation can be observed until the first 2 mol of Ca^{2+} is added. As the molar ratio of Ca^{2+} to CaM is increased from 2 to 4, this resonance splits into two peaks, accompanied with upfield shifts.

The chemical shift data for His-107, Tyr-138, and Tml-115 of scallop testis CaM at pH 8.8 and at 23 °C and at three molar ratios of Ca^{2+} to CaM of 0, 2, and 4 are summarized in Table I.

Tyr-99 of Pig Brain Calmodulin. Assignments of resonances of Tyr-99 of pig brain CaM in the Ca^{2+}-free state have been made in the preceding paper. Assignments in the presence of 2 mol of Ca^{2+}/mol of CaM and in the presence of 4 mol of Ca^{2+}/mol of CaM were performed by spin-decoupling experiments and NOE difference spectroscopy. Upon addition of the first 2 mol of Ca^{2+}, the resonance at 6.82 ppm due to δ protons of Tyr-99 in the Ca^{2+}-free state shifts slightly upfield to 6.79 ppm, and the resonance of the ϵ protons shifts from 7.19 to 6.95 ppm (data not shown). In the Ca^{2+}-saturated state, the resonance of the δ protons overlaps with the resonance of δ protons of Phe-X, which merge at molar ratios above 2. The chemical shift data of Tyr-99 of pig brain CaM at pH 8.8, at 23 °C, and at three molar ratios of Ca^{2+}/CaM of 0, 2, and 4 are also listed in Table I.

Nuclear Overhauser Enhancement. Figure 5 shows NOE difference spectra at three different Ca^{2+} contents when the resonance of Try-138 δ protons is irradiated. Appreciable NOE effects can be observed on the resonance of Phe-B δ protons in the presence of 2 mol of Ca^{2+}/mol of CaM and in the presence of 4 mol of Ca^{2+}/mol of CaM, but in the absence of Ca^{2+}, the resonance of Phe-B δ protons exhibits no effects.

Figure 7 shows NOE difference spectra in the Ca^{2+}-free state when the resonance of Ile-A γ-methyl (peak f) and the resonance of Ile-A δ-methyl (peak i) are irradiated. Relatively large NOE effects can be observed on resonances of other protons of Ile-A and also on the resonances of γ-methyl protons of Val-A (peak K). Furthermore, the ring resonances of both Phe-A and Phe-B and also Phe-Z exhibit NOE effects, but the resonances of Try-138 are not affected.

Figure 8 shows aromatic parts of NOE difference spectra in the Ca^{2+}-free state and in the Ca^{2+}-saturated state when the resonance of the γ-methyl of Val-A (peak k) is irradiated. In the Ca^{2+}-free state, the resonance of Phe-Z exhibits a relatively large NOE effect while no appreciable effects can be observed on other aromatic residues. On the other hand, in the Ca^{2+}-saturated state, only the resonances of δ and ϵ protons of Tyr-138 are affected by the irradiation of the resonance of the γ-methyl of Val-A. These results were confirmed by observing the NOE effects when each of the resonances of Tyr-138, Phe-A, and Phe-B is irradiated.

Discussion

The dependence of chemical shift on the Ca^{2+} content shown in Figure 3 indicates that the resonances can be approximately classified into three groups. Krebs & Carafoli (1982) have also reported that the Met resonances in bovine brain CaM can be classified into three groups, but their classification is different from ours. The resonances classified into group I vary only in the range of 0–2 mol of Ca^{2+}, and the change in resonances of group II can be observed only at molar ratios above 2. The resonances of group III alter over the whole range of Ca^{2+} content from 0 to 4.

Group I includes the resonances of Tyr-138, His-107, Tml-115, Tyr-99, Phe-B, Ile-A, Val-B, and three singlet methyls. As evidenced by the existence of two kinds of resonances, two species of Ca^{2+}-free and -bound CaM coexist in

solution below 2 mol of Ca^{2+}/mol of CaM, suggesting that the exchange between the two species is slow compared to the NMR time scale. Above 2 mol of Ca^{2+}, no Ca^{2+}-free CaM resonances are evident, indicating that the first conformational transition is completed by the binding of the first two Ca^{2+}. The fact that there is no change in chemical shift of the resonances of group I in the range of Ca^{2+} content from 0 to 2 implies that the species of the CaM–Ca^{2+} complex present in this range of Ca^{2+} content is only CaM–$(Ca^{2+})_2$; no CaM–$(Ca^{2+})_1$ complex is formed. It is indicated that the binding of the first two Ca^{2+} to CaM is strongly cooperative, in agreement with the result of a previous equilibrium dialysis study (Crouch & Klee, 1980).

Seamon (1980) reported on the basis of his assignments that the ϵ protons of Tyr-138 are affected by a relatively fast exchange of Ca^{2+} at a binding site while the δ protons are affected by a slow conformational transition between two stable conformations of the proteins. The present results however indicate that both δ and ϵ protons of Tyr-138 exhibit a slow exchange rate and are at variance with Seamon's results. This is because our assignments of the resonances of Tyr-138 in the intermediate range of Ca^{2+} content differ from his assignments.

Tyr-138 in bovine brain CaM has been reported to have a high pK value of 11.9 both in the absence and presence of Ca^{2+} (Richman & Klee, 1978). Seamon (1979) and Kreb & Carafoli (1982) showed such a high pK value (above 11.5) from the NMR measurements. This was also confirmed by the present study. Tyr-138 of scallop testis CaM has a high pK value of 12 in both the absence and presence of Ca^{2+}, suggesting that Tyr-138 is situated in a hydrophobic environment, as described by other authors (Richman & Klee, 1978; Seamon, 1980). Tyr-150 of cardiac TnC also has a high pK value of 11.4 in the presence of Ca^{2+} (Hincke et al., 1981a) and shows a very similar spectral change with increasing Ca^{2+} content as does Tyr-138 of CaM. It is of interest to note the similarity in spectral change between Tyr-138 of CaM and Tyr-150 of cardiac TnC.

H2 and H4 protons of His-107 in group I are also affected by a slow exchange rate in the range of Ca^{2+} content from 0 to 2 mol. A similar slow-exchange phenomenon has been observed on the H2 and H4 protons of His-125 of skeletal TnC in this range of Ca^{2+} content (Levine et al., 1977). His-107 of CaM is located at a position adjacent to binding site III. His-125 of skeletal TnC is located between binding sites III and IV and occupies a position homologous to that of Tml-115 of CaM. The present results as well as Seamon's results indicate that the ϵ-trimethyl resonance of Tml-115 also reflects the slow conformational transition between the two species in this stage of Ca^{2+} binding.

Krebs & Carafoli (1982) indicated that the resonance of His-107 H2 protons observed at pH 9.5 and at 30 °C becomes broad with increasing Ca^{2+} content, suggesting that His-107 is in an intermediate exchange state. The difference between the results obtained by these authors and those of the present study can be accounted for different experimental conditions. Some important differences between the two are temperature, pH, and the observing frequency. We found that the two resonances of His-107 H2 protons observed at a molar ratio of Ca^{2+}/CaM of 1 at pH 8.8 and at 23 °C collapse into a single broad peak when observed at pH 8.8 and at 40 °C. This is caused by the increase in exchange rate between Ca^{2+}-free and -bound states with increasing temperature.

The chemical shifts of Tyr-99 in the absence and presence of Ca^{2+} are in agreement with those reported by Krebs & Carafoli (1982) but not with those by Seamon (1980). The resonances of Tyr-99 are perturbed only as the first two Ca^{2+} are added. This behavior is similar to those of Tyr-109 of skeletal TnC (Levine et al., 1977) and Tyr-111 of cardiac TnC (Hincke et al., 1981a); the tyrosines occupy a position homologous to Tyr-99 of CaM. These homologous tyrosines exhibit normal pK values, suggesting that these are exposed to water in both the absence and presence of Ca^{2+}.

Furthermore, group I includes the resonances of Phe-B, Ile-A, and Val-B, and also the resonances of Met residues at 2.14 (S1), 1.87 (S9), and 1.47 ppm (S10) (refer to the preceding paper for the designation; Ikura et al., 1983). Krebs & Carafoli (1982) assigned the resonance at 2.14 ppm (S1) to the N-acetyl group. All these resonances are dominated by the slow-exchange process.

Tyr-138, His-107, Tml-115, and Tyr-99 are located in the neighborhood of Ca^{2+}-binding sites III and IV. The resonances of these residues are perturbed mainly by the binding of the first two Ca^{2+}. Thus, we conclude that sites III and IV are the high-affinity sites, in agreement with Seamon's conclusion. It has been reported that the high-affinity sites of TnC are sites III and IV (Sin et al., 1978). It is worthwhile to note the similarity in the high-affinity sites between CaM and TnC as well as other similarities in spectral behaviors.

As described before, the results of Tb^{3+} luminescence spectroscopy (Wallace et al., 1982; Wang et al., 1982) and Ca^{2+}/Gd^{3+} exchange experiments by ^1H NMR (Krebs & Carafoli, 1982) are at variance with the present results. It seems that the binding mechanism of trivalent cations Tb^{3+} and Gd^{3+} is different from that of divalent cation Ca^{2+}.

Group II includes the resonances of Phe-X (peak e), ring current shifted methyl (peak o) and ring current shifted α-methine (peak p), and four Met. These resonances undergo change in chemical shift in the range of Ca^{2+} content from 2 to 4. Most of these resonances reflect a fast exchange rate. The abnormal chemical shifts suggest that these residues are situated in hydrophobic environments. The second conformational transition reflected by these resonances is induced by the binding of Ca^{2+} only to the low-affinity sites. It is of interest to note that the resonance of the δ protons of Tyr-138 slightly shifts downfield from 6.33 to 6.35 ppm in the course of the second conformational change. The methyl resonances of Met residues at 2.13 (S2), 2.10 (S3), and 2.00 ppm (S4 and S5) seem to be classified into group II and are affected by the fast exchange rate.

The resonances of group III are affected by the binding of Ca^{2+} to both the high- and low-affinity sites. This group includes the resonances of Phe-A, high-field shifted methyl (peak m), Thr-143, and two Met. Phe-A (peak d) undergoes a transition over the whole range of Ca^{2+} content and exhibits a fast exchange rate. Peak m, a methyl resonance shifted upfield due to the ring-current effect, is affected by a slow exchange rate upon addition of the first two Ca^{2+}, while above 2 mol of Ca^{2+}, this resonance is characterized by a fast exchange process. Thus, it may be considered that peak m exhibits the combined properties of group I and group II. Moreover, the resonance of γ-methyl of Thr-143 (peak q) is affected both by the first and second conformational transitions. Resonances of methyl groups of Met residues at 1.99 (S6) and at 1.97 ppm (S7) may be included in group III. The methyl groups of these Met residues are reflected by the fast exchange process in the whole range of Ca^{2+} content 0–4.

The results of the previous study (Ikura et al., 1983) and the present NOE experiments indicate that in the Ca^{2+}-free state Phe-A, Phe-B, and Ile-A are close to each other and also

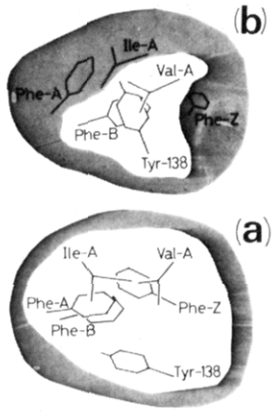

FIGURE 9: Spatial arrangements of amino acids in the vicinity of Tyr-138: (a) in the absence of Ca^{2+}; (b) at molar ratios of Ca^{2+}/calmodulin above 2. The NOE experiments were not carried out on the resonances of the residues shown in the shadowed area.

that Ile-A, Val-A, and Phe-Z are located near by to each other. Tyr-138 is distant from these residues in the Ca^{2+}-free state. On the other hand, it can be seen from the results of NOE experiments that Tyr-138 approaches Phe-B and Val-A in the course of the first conformational transition. This is in contrast to the results reported by Krebs & Carafoli (1982) that Tyr-138, Tyr-99, and phenylalanine(s) are close to each other in both the Ca^{2+}-free and Ca^{2+}-saturated states. The large upfield shifts of the resonances of Tyr-138 in the first stage of Ca^{2+} binding are accounted for by the ring-current effect due to the phenyl ring of Phe-B. The environments around Tyr-138 in the absence and presence of Ca^{2+} are schematically depicted in Figure 9. It can be seen that hydrophobic amino acids form a cluster in the vicinity of Tyr-138 in the presence of Ca^{2+}, in agreement with the fact that Tyr-138 has the high pK value. The local environment around Tyr-138 in the absence of Ca^{2+}, however, could not be elucidated in the present study. The results of the UV absorption difference spectrum of CaM (Yazawa et al., 1980; Seamon & Moore, 1980) suggested that the environment around Tyr-138 is more hydrophobic in the absence of Ca^{2+} than in the presence of Ca^{2+}. It is suggested that in the absence of Ca^{2+} Tyr-138 is situated in a more hydrophobic environment constituted by some aliphatic residues that may be different from those shown in Figure 9a.

The three phenylalanines, Phe-A, Phe-B, and Phe-Z, which are in relatively close proximity to Tyr-138, would be tentatively assigned to any of Phe-89, Phe-92, and Phe-141, as judged from the predicted structure of CaM (Kretsinger, 1980b). Two Met residues that are affected by the Ca^{2+} binding to the high-affinity sites, S9 and S10, may be attributed to Met-109 and Met-124.

If one takes into acount an association constant for the high-affinity sites of 4×10^5 M^{-1} (Yazawa et al., 1978) and uses the smallest separation between two resonances of Tml-115 ϵ-trimethyl protons in the first conformational transition, the off-rate constant of the first two Ca^{2+} from CaM is estimated to be less than 50 s^{-1}, and consequently, the on-rate would be less than 2×10^7 s^{-1} M^{-1}. It is, therefore, likely that the rate of Ca^{2+} binding to the high-affinity sites is determined by a diffusion-controlled process. The rate of Ca^{2+} release from the high-affinity sites is slow. These results are consistent with the results of a previous rapid kinetic study (Malencik et al., 1980), in which the off-rate was estimated to be 10 s^{-1}.

On the other hand, several resonances experience the fast exchange process in the second conformational transition. The Ca^{2+} off-rate constant at the low-affinity sites is also estimated to be more than 600 s^{-1}, using the separation of low field shifted α protons (Figure 4, peak p).

The present study indicated a marked similarity in structural change between CaM and TnC during the binding of Ca^{2+} to the high-affinity sites. Since the two proteins function in a different manner, however, some difference in spectral behavior should be expected. The resonances of cardiac TnC, which change during the binding of Ca^{2+} to the low-affinity site, correspond to the resonances of the ring current shifted methyl (peaks f and g in Figure 3) and the uniquely high field shifted Phe (Phe-A and Phe-B) for CaM; most of the CaM resonances belong to group I. It might be possible that these differences are related to the difference in function between CaM and TnC. Hincke et al. (1981a) suggested for TnC that the high-affinity sites play mainly a structural role while the low-affinity site(s) is (are) the regulatory one(s). It is likely that the resonances of group II reflect the most important structural change related to the function of CaM.

In summary, (i) the resonances of CaM can be classified into three groups with respect to the dependence of spectra on the content of Ca^{2+}. Group I (affected by the binding of Ca^{2+} to the high-affinity sites) includes the resonances of Tyr-138, His-107, Tml-115, Tyr-99, Phe-B, Ile-A, Val-B, two Met, and the N-acetyl group. Group II (affected by the binding of Ca^{2+} to the low-affinity sites) includes the resonances of Phe-X, a high field shifted methyl, a low field shifted α-methine, and four Met. Group III (affected by the binding of Ca^{2+} both to the high- and low-affinity sites) includes the resonances of Phe-A, a high field shifted methyl, Thr-143, and two Met. (ii) The present data indicate that sites III and IV are the high-affinity Ca^{2+}-binding sites. (iii) Tyr-138 is situated in hydrophobic environments in both the absence and presence of Ca^{2+}, as evidenced by the high pK values. The NOE experiments demonstrate that in the Ca^{2+}-free state Tyr-138 is distant from Phe-A, Phe-B, Phe-Z, Val-A, and Ile-A, although the latter five amino acid residues are close together. Upon binding of Ca^{2+} to the high-affinity sites, Phe-B and Val-A approach to Tyr-138. (iv) The off-rate of Ca^{2+} from the high-affinity sites is slower than 50 s^{-1}. On the other hand, the off-rate from the low-affinity sites is faster than 600 s^{-1}. (v) Many similarities in structural change between CaM and TnC are recognized in the course of the binding of Ca^{2+} to the high-affinity sites, but the structural change of CaM associated with the binding of Ca^{2+} to the low-affinity sites is somewhat different from that of TnC.

Registry No. Calcium, 7440-70-2; L-tyrosine, 60-18-4; (*S*)-ϵ-trimethyllysine, 19253-88-4; L-histidine, 71-00-1; L-phenylalanine, 63-91-2; L-isoleucine, 73-32-5; L-valine, 72-18-4.

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Insulin Receptor: Insulin-Modulated Interconversion between Distinct Molecular Forms Involving Disulfide–Sulfhydryl Exchange†

Joseph M. Maturo III,* Morley D. Hollenberg, and Linda S. Aglio*

ABSTRACT: When insulin receptor was isolated from human placenta membranes by a sequential chromatographic procedure (method I) that did not expose the receptor to insulin, the purified receptor (about 2000-fold purification) was eluted on columns of Sepharose 6B in a volume corresponding to a K_{av} of 0.31 (R_I form of the receptor, with an apparent Stokes radius of about 7.2 nm). In contrast, the placenta receptor isolated by insulin–agarose affinity chromatography (method II) was eluted on Sepharose 6B columns with a K_{av} of 0.53 (R_{II} form of the receptor, with an apparent Stokes radius of about 3.8 nm). When receptor prepared by method I was exposed to insulin, the R_I form of the receptor was converted to the R_{II} form; this insulin-mediated change in the receptor elution behavior was mimicked by dithiothreitol (0.1 mM) treatment. In concert with the insulin-mediated conversion of the R_I to the R_{II} form of the receptor, we observed insulin-stimulated incorporation of ^{3}H-labeled *N*-ethylmaleimide (^{3}H-NEM) into the receptor preparation. Both the insulin-mediated interconversion of the receptor from the R_I to the R_{II} form and the insulin-stimulated incorporation of ^{3}H-NEM were dependent on insulin concentration; the concentration dependence indicated that a half-maximal effect occurred at about 1 nM insulin. Both ^{125}I-labeled receptor, converted to the R_{II} form in the presence of insulin, and the R_{II} form of the receptor labeled with ^{3}H-NEM in the presence of insulin were bound by anti-insulin receptor immunoglobulin, obtained from an individual with severe insulin resistance. Exposure of the R_I form of the receptor to insulin–agarose also led concurrently to the conversion of the receptor from the R_I to the R_{II} form and to the incorporation of ^{3}H-NEM. The R_{II} form of the receptor obtained by method II from insulin–agarose could be converted back to the R_I form by treatment with oxidized glutathione. The experiments with purified receptor preparations were complemented by studies with particulate membrane preparations cross-link labeled with ^{125}I-labeled insulin both at low (0.5 ng/mL) and comparatively high (25 ng/mL) insulin concentrations. Solubilization and immunoaffinity purification of receptor, cross-link labeled in the membranes with 0.5 ng/mL ^{125}I-labeled insulin, yielded material that on Sepharose 6B columns behaved like the R_I form of the receptor. In contrast, immunoaffinity-isolated receptor, cross-link labeled in the membranes with 25 ng/mL ^{125}I-labeled insulin, behaved on Sepharose 6B columns like the R_{II} form of the receptor; receptor cross-link labeled at the high (25 ng/mL) insulin concentration (but not at 0.5 ng/mL insulin) simultaneously incorporated ^{3}H-NEM. Our results indicate that both in membranes and in purified soluble receptor preparations, insulin causes an interconversion of its receptor from one hydrodynamic form to another by a process involving a disulfide–sulfhydryl exchange.

There has recently been considerable progress in understanding the molecular structure of the receptor for insulin [current views summarized in volume edited by Andreani et al. (1981)]. One salient feature of the heterodimeric structure proposed for the insulin receptor (Jacobs et al., 1980; Massagué et al., 1980) relates to the presence of a number of disulfide bonds that stabilize the proposed oligomeric $(\alpha\beta)_2$ structure. It is further evident that the entire receptor structure, as it exists in the cell membrane and in crude detergent extracts of cell membranes, may comprise not only the $(\alpha\beta)_2$ recognition oligomer that has been the species of intensive investigation but may also include other closely associated polypeptide chains. Evidence for the presence of such receptor-

† From the Biology Department, C. W. Post College, Greenvale, New York 11548 (J.M.M. and L.S.A.), and the Endocrine Research Group, Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Calgary, Calgary, Canada T2N 4N1 (M.D.H.). *Received August 27, 1982.* This work was supported in large part by grants-in-aid from C. W. Post College and in some part by a grant (to M.D.H.) from the Canadian Medical Research Council. L.S.A. was supported by a Student National Institutes of Health fellowship granted to the Albert Einstein College of Medicine, Bronx, New York, NY.

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