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# HIGH FIELD PROTON NMR STUDIES OF TRYPTIC FRAGMENTS OF CALMODULIN: A COMPARISON WITH THE NATIVE PROTEIN

Ann Aulabaugh, Walter P. Niemczura and William A. Gibbons

Department of Biochemistry, College of Agricultural and Life Sciences
University of Wisconsin-Madison

Department of Pharmaceutical Chemistry, School of Pharmacy
University of London, London, England

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Addition of the 600 MHz proton magnetic resonance (PMR) spectra of two calmodulin tryptic fragments, residues 1-77 and 78-148, both in the apo- and calcium-bound forms, yielded, to a first order, the corresponding spectrum of intact apo- and calcium-bound calmodulin, respectively. This plus additional data permitted: 1) assignments of resonances to a particular sequence 2) the hypothesis that the secondary and tertiary structures and the calcium binding properties are similar in the fragments and intact calmodulin 3) assignment of the two high affinity calcium sites to the sequence 78-148.

Calmodulin (CAM), a ubiquitous eukaryotic calcium binding protein, possesses a single polypeptide chain of 148 residues that has four calcium binding sites, and is chemically modified by acetylation of the N-terminus and methylation of Lys-115. Despite this simplicity, calmodulin exerts a profound effect on a large variety of biological processes by modulating intracellular enzymatic reactions and calcium dependent processes. (For reviews see refs. 1-4). A great deal of structural, kinetic and thermodynamic experiments have been performed to explain how this single small protein can control or modulate many intracellular events. Published structural studies have utilized ultraviolet spectroscopy (6-8); circular dichroism (6,7,9-11); tyrosine fluorescence (12,13); terbium luminescence (13-17; proton, $^{113}Cd$, $^{45}Ca$ and $^{23}Na$ nuclear magnetic resonance spectroscopy (5,17-31); Raman spectroscopy (32) and X-ray crystallography (33). Summaries of the research on the metal-dependent structural and conform-

Abbreviations: CAM, calmodulin; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; TR1C, calcium-bound calmodulin tryptic fragment, residues 1-77, containing calcium binding sites I and II; TR2C, calcium-bound calmodulin tryptic fragment, residues 78-148, containing calcium binding sites III and IV; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; ppm, part per million; UV, ultraviolet.

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ational dynamics of CAM and its association with enzymes have appeared (4,5).

CAM is in the molecular weight range accessible to proton magnetic resonance (PMR) techniques but only a limited number of assignments and conclusions have been forthcoming. Here we report the use of highly purified tryptic fragments of CAM to simplify the PMR spectral assignments, to clarify domain-domain interactions and the manner of calcium binding to domains. Similar studies by $^{23}Na$ and $^{113}Cd$ magnetic resonance have appeared (30,31).

## MATERIALS AND METHODS

Calmodulin was prepared from fresh bovine testicles as previously described (18) except EDTA was omitted early during the chromatography steps. A modification of the procedure of Drabikowski et al (34) was used to prepare calmodulin tryptic fragments. Calmodulin (1 mg/ml) was treated with TPCK-treated trypsin (enzyme/substrate, 1/120, w/w) for 20 or 40 minutes at 20° C in 20 mM ammonium carbonate and excess calcium. Soybean trypsin inhibitor was added to terminate the enzymatic reaction, followed by treatment with Chelex-100 resin to remove calcium and then fractionation of the supernatant by ion exchange on a DEAE-52 column using a salt gradient, 0.1 to 0.35 M NaCl in 5 mM potassium phosphate buffer, pH = 7.5. After lyophilization, samples were desalted on a Sephadex G25 column and decalcified on a Chelex-100 column. The pH of the eluent was adjusted to pH = 7.5 with 0.1 M NaOH. Protein and peptide purity were monitored by native and 8 M urea 15% polyacrylamide gels, ultraviolet spectroscopy, and proton NMR spectroscopy.

600 MHz proton NMR spectra were run at the NIH National Facility for Biochemical Studies at Carnegie-Mellon University. Spectra were acquired in the Fourier mode using quadrature detection, with a pulse width of 19 usec (corresponding to a 90° flip angle), a sweep width of 1.25 sec, and a delay between scans of 1 sec. Typically, each spectrum represents the coaddition of 1000 free induction decays (FID). The resulting FID was apodized with a decreasing exponential (time constant of 0.7 sec), Fourier transformed and phase corrected.

## RESULTS AND DISCUSSION

Fragment Characterization. The chromatogram from a 40 minute limited tryptic digestion of fully calcium-bound bovine testes calmodulin (CAM), yielded two major fractions designated TR1C' and TR2C and two minor fractions, TR2C' and CAM, Figure 1. As expected, decreasing the incubation time to 24 minutes decreased the total yield of tryptic fragments but produced a new peak, TR1C, eluting before TR1C'. Amino acid analysis confirmed the identity of the tryptic fragments

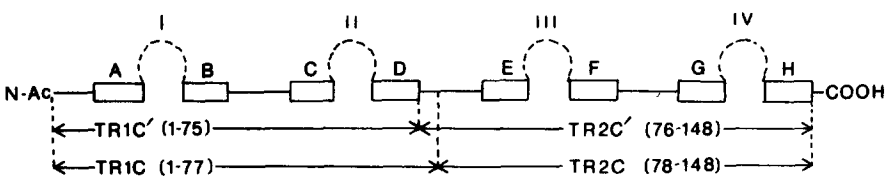


Figure 1: Diagrammatic representation of the primary sequence of calmodulin including the calcium binding sites I-IV, proposed alpha helices, A-H, and the tryptic fragments.

shown above. UV spectroscopy and proton NMR spectroscopy at 600 MHz confirmed the sequence assignments from analysis of their relative Phe and Tyr residue content, and the Met content in the NMR spectra.

Comparison of proton magnetic resonance spectra of the aromatic residues of TR1C with that of TR2C confirmed the above (Figure 2A and 2B). Addition of the spectra of the fragments, Figure 2C, yielded, to a first order, the spectrum of apoCAM itself, Figure 2D. Furthermore, the spectrum of TR1C, Figure 2A, con-

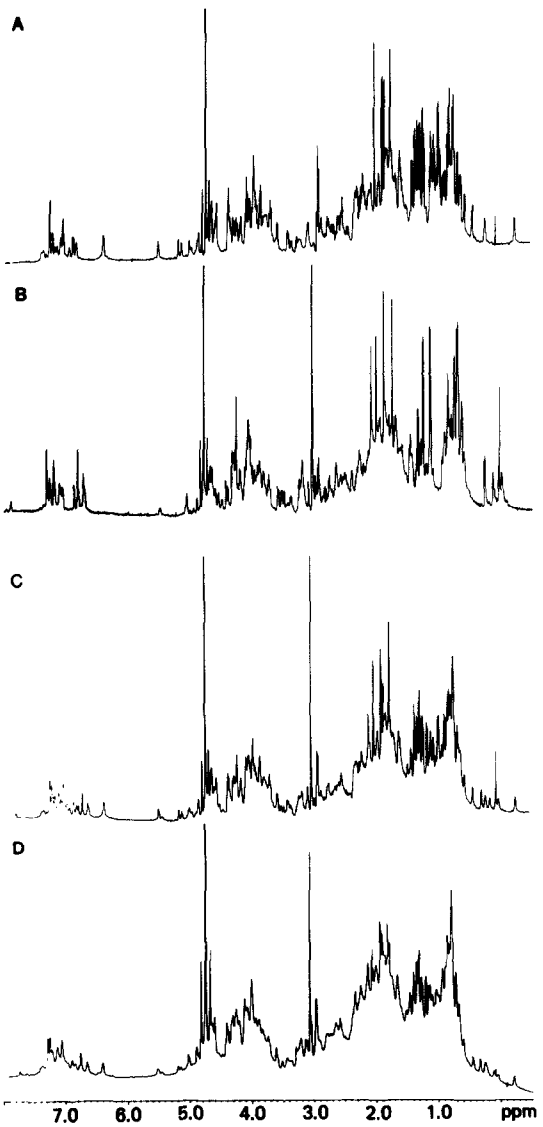


Figure 2: Aliphatic and aromatic regions of the calcium free spectrum of (A) TR1C; (B) TR2C1 (C) computer sum of TR1C and TR2C spectra; (D) calmodulin; [fragments] = $2 \times 10^{-3}$ M, [calmodulin] = $1 \times 10^{-3}$ M, pH = 7.5.

tains twenty-five aromatic protons of the five Phe residues and no Tyr aromatic resonances, while Figure 2B integrated for three Phe, two Tyr, and one His residues. Similar additivities were possible for residues such as the $\varepsilon$-$CH_3$ groups of the Met residues and high field-shifted methyl resonances, Figure 2.

Resonance assignments to domains and domain-domain interactions. The additivity of the TR1C and TR2C NMR spectra and the similarity between the addition spectrum and that of CAM permitted us to assign some proton resonances to certain halves of the CAM molecule and conclude the calcium binding domains of the intact protein are the same as those of the TR1C and TR2C fragments.

Tables 1 and 2 were derived by comparing the upfield-shifted methyl peaks and the aromatic peaks of the CAM spectrum with the TR1C and TR2C spectra. An important corollary is that the majority of chemical shifts in TR1C are not affected by the residues of TR2C and vice versa. It follows that in apoCAM domains I and II do not interact to any major extent with domains III and IV.

This additivity of domains I and II with domains III and IV is reflected in three classes of anomalously shifted resonances: 1) high field-shifted resonances (Table 1, Figure 2) 2) aromatic ring protons (Table 2, Figure 2) 3) backbone alpha protons (Figure 2, from 4.7 to 5.5 ppm). Conformational inferences can be drawn from these particular observations since a principal cause of anomalously shifted proton resonances is proximity to Phe, Tyr, and Trp rings. The additivity of the spectra of apoTR1C and TR2C therefore proves that the secondary and tertiary conformations of the fragment TR1C, containing domains I and II,

TABLE 1. UPFIELD-SHIFTED METHYL PEAK POSITIONS[a]

| Fragment | Calcium | Peak Positions, ppm | | | | | | |
|---|---|---|---|---|---|---|---|---|
| TR1C | - | 0.44 | | 0.24 | | | | -0.24 |
| TR2C | - | | 0.30 | | 0.17 | 0.07 | 0.03 | |
| CAM | - | 0.42 | 0.31 | 0.24 | 0.22 | 0.12 | 0.04 | -0.26 |
| TR1C | + | 0.56 | | | 0.37 | 0.23 | | |
| TR2C | + | | 0.46 | 0.39 | | 0.23 | | |
| CAM | + | 0.57 | 0.49 | | 0.37 | 0.23 | | |

a Each signal corresponds to three protons

TABLE 2. AROMATIC PEAK POSITIONS OF FRAGMENTS AND INTACT CAM

| ApoTR1C | ApoTR2C | ApoCAM | (ppm) | $Ca_2$TR1C | $Ca_2$TR2C | $Ca_4$CAM | (ppm) |
|---|---|---|---|---|---|---|---|
| | 7.78 | 7.70 | His-107 | | 7.64 | 7.73 | His-107 |
| 7.36 | | 7.35 | Phe | | 7.37 | 7.34 | Phe |
| 7.23 | 7.22 | 7.22 | Phe | 7.32 | 7.30 | 7.29 | Phe |
| | 7.18 | 7.19 | Phe | 7.27 | 7.24 | 7.24 | Phe |
| 7.17 | | 7.17 | Phe | 7.18 | | 7.18 | Phe |
| 7.15 | | 7.15 | Phe | 7.16 | | 7.14 | Phe |
| | 7.06 | 7.08 | Phe | 7.09 | 7.07 | 7.09 | Phe |
| 7.00 | 6.98 | 7.00 | Phe | 6.96 | 6.97 | 6.96 | Phe |
| | 6.95 | 6.95 | Phe | | 6.92 | 6.93 | Phe |
| 6.92 | | 6.92 | Phe | 6.88 | 6.87 | 6.87 | Phe, His-107, Tyr-99 |
| 6.85 | | 6.87 | Phe | | 6.85 | 6.84 | Tyr-99 |
| 6.80 | | 6.82 | Phe | 6.70 | 6.69 | 6.68 | Tyr-99, Phe (TR1C) |
| | 6.79 | 6.76 | His-107 | 6.56 | 6.56 | 6.54 | Phe (TR1C), Phe (TR2C) |
| | 6.73 | 6.73 | Tyr-99 | | 6.45 | 6.44 | Tyr-138 |
| | 6.64 | 6.63 | Tyr-138 | | 6.23 | 6.23 | Tyr-138 |
| 6.37 | | 6.37 | Phe | | | | |

is independent, to a first order, of the sequence and conformation of TR2C, containing domains III and IV, and vice versa.

Localization of secondary and tertiary structural changes due to calcium. The calcium-bound forms of TR1C and TR2C were examined to determine whether they were analogous to $Ca_4$CAM. The relevant calcium spectra in Figure 3 plus the data summarized in Tables 1 and 2 confirmed that: 1) considerable structural homology is retained in the calcium-bound fragments, as measured by the additivity of the aromatic, high-field shifted methyl and other anisotropically shifted resonances 2) conformational integrity and homology is maintained in the calcium fragments 3) the ability of the sequences to bind calcium and the ligand sites are retained in the fragments 4) calcium affinity is reduced and 5) the secondary and tertiary structure of calcium-bound residues 1-77 are not significantly affected by residues 78-148. Thus, conformationally CAM has two halves

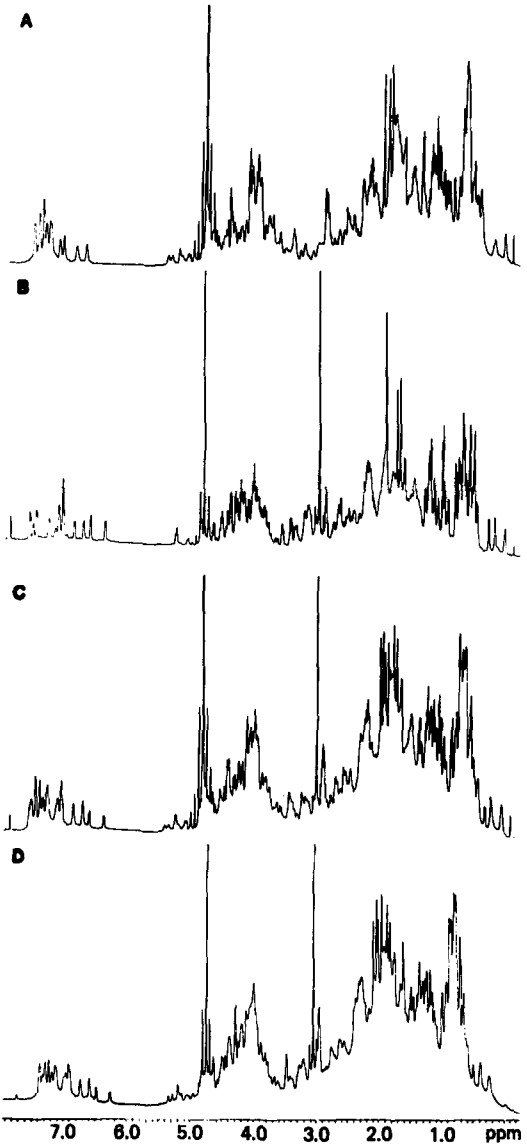


Figure 3: Aliphatic and aromatic regions of the calcium bound spectra of (A) TR1C; (B) TR2C; (C) computer sum of TR1C and TR2C spectra; (D) calmodulin; [fragments] = $2 \times 10^{-3}$M, [calmodulin] = $1 \times 10^{-3}$M, pH = 7.5.

which behave relatively independently in their apo and in their calcium-bound form, a conclusion supported by a recent crystallographic study (33).

Further proof of this and insight into the nature of the conformational changes that occur during calcium binding were obtained by comparing spectral changes seen during calcium titrations. The chemical shift changes observed for TR2C were relatively similar to those observed with the intact CAM, e.g. the trimethyllysine-115 peak shifted upfield by 0.02 ppm in both cases with concom-

itant changes in the spectra attributable to Tyr-99 and Tyr-138, to the low field shifted alpha proton resonances and to the high field-shifted methyl protons. Thus the conformational changes that occur upon calcium binding to residues in the 78-148 fragment are predominantly due to localized changes in the micro-environment within the fragment examined and not to changes in the more remote residues within the 1-77 sequence.

Upon comparison of previously reported CAM calcium titration data measured by proton NMR (18) with the calcium bound fragments, domains III and IV are identified as the high affinity sites, in agreement with a $^{113}Cd$ NMR study of the fragments (31). Proton NMR work in this lab (35) comparing the spectral changes induced upon titration of CAM with calcium, cadmium and lanthanide indicates that the binding mechanism and order is different for the divalent ions compared to the trivalent ion.

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