Adman, E. T., Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1978) *J. Mol. Biol. 123*, 35–47.
Cookson, D. J., Hayes, M. T., & Wright, P. E. (1980) *Biochim. Biophys. Acta 591*, 162–176.
Corin, A. F. (1981) Ph.D. Thesis, Columbia University.
Dwek, R. A. (1973) *NMR in Biochemistry*, Clarendon Press, Oxford.
Eley, C. G. S., Moore, G. R., Williams, G., & Williams, R. J. P. (1982) *Eur. J. Biochem. 124*, 295–303.
Farver, O., & Pecht, I. (1981) *Isr. J. Chem. 21*, 13–17.
Farver, O., Blatt, Y., & Pecht, I. (1982) *Biochemistry 21*, 3556–3561.
Freeman, H. C. (1981) in *Coordination Chemistry* (Laurent, J. P., Ed.) Vol. 21, p 29, Pergamon Press, Oxford.
Goldberg, M. (1979) Ph.D. Thesis, The Weizmann Institute of Science, Rehovot, Israel.
Goldberg, M., & Pecht, I. (1976) *Biochemistry 15*, 4197–4208.
Handford, P. M., Hill, H. A. O., Lee, W. K., Henderson, R. A., & Sykes, A. G. (1980) *J. Inorg. Biochem. 13*, 83–88.
Hill, H. A. O., & Smith, B. (1979) *J. Inorg. Biochem. 11*, 79–93.
Hopfield, J. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 195–202.
Hopfield, J. J., & Urgubil, K. (1982) in *Electron Transport and Oxygen Utilization* (Ho, C., Ed.) pp 81–84, Elsevier, New York.
Lappin, A. G., Segal, M. G., Weatherburn, D. C., Henderson, R. A., & Sykes, A. G. (1979) *J. Am. Chem. Soc. 101*, 2302–2306.
McCray, J. A., & Kihara, T. (1979) *Biochim. Biophys. Acta 548*, 417–426.
Mitra, S., & Bersohn, R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6807–6811.
Rosen, P., & Pecht, I. (1976) *Biochemistry 15*, 775–786.
Rosen, P., Segal, M., & Pecht, I. (1981) *Eur. J. Biochem. 120*, 339–344.
Segal, M. G., & Sykes, A. G. (1978) *J. Am. Chem. Soc. 100*, 4585–4592.
Stellwagen, E., & Shulman, R. G. (1973) *J. Mol. Biol. 80*, 559–573.
Urgubil, K., & Bersohn, R. (1977) *Biochemistry 16*, 3016–3023.

Metal Ion and Drug Binding to Proteolytic Fragments of Calmodulin: Proteolytic, Cadmium-113, and Proton Nuclear Magnetic Resonance Studies†

Eva Thulin, Anita Andersson, Torbjörn Drakenberg, Sture Forsén, and Hans J. Vogel*

ABSTRACT: Tryptic fragmentation of Ca^{2+}-saturated calmodulin (CaM) takes place mainly at Lys-77; however, proteolysis can occur instead at Arg-74 or Lys-75. This cleavage pattern results in the production of three peptides each of the amino- and carboxy-terminal halves of CaM of slightly different length. A purification scheme for the three carboxy-terminal half-peptides is reported. Proton nuclear magnetic resonance (^1H NMR) studies of peptides comprising the amino- or carboxy-terminal half of CaM reveal the great structural similarity between these two proteolytic fragments and the intact protein. Since this was observed for the apoprotein as well as the Ca^{2+}-saturated protein, this means that the two halves of the protein are independently folded. A comparison of the changes in the ^1H NMR spectra observed for the intact protein and the fragments upon addition of Ca^{2+} clearly identified sites III and IV as the two high-affinity binding sites. Furthermore, addition of Ca^{2+} or Cd^{2+} induces qualitatively similar changes in the spectra, thus indicating that Cd^{2+} is a reliable replacement for Ca^{2+} in these studies. Subsequent ^{113}Cd NMR studies of trifluoperazine (TFP) binding to tryptic and thrombic fragments of calmodulin revealed the presence of two distinct drug binding sites, one located in the amino-terminal half and one located in the carboxy-terminal half. The spectral changes, induced upon addition of the antipsychotic drug, were similar to those observed upon binding of TFP to intact calmodulin. The strongest TFP binding site is located in the carboxy-terminal half.

Hormonal or nerve impulses can cause the level of cytosolic Ca^{2+} in eukaryotic cells to rise from 10^{-7} to 10^{-5} M. During such an influx, the metal ion binds to regulatory calcium binding proteins like troponin C (TnC)[1] and calmodulin (CaM) which in response undergo large conformational changes. The completion of the amino acid sequence of CaM revealed its high homology to skeletal TnC (Watterson et al., 1980); both proteins contain four calcium binding domains each arranged in a continuous helix–loop–helix sequence, often called the EF hand (Kretsinger, 1976). TnC is the regulatory calcium binding subunit of the troponin complex that triggers contraction of striated muscles (McCubbin & Kay, 1980). Its soluble analogue CaM exposes hydrophobic regions upon Ca^{2+} binding (LaPorte et al., 1980; Tanaka & Hidaka, 1980; Vogel

† From the Department of Physical Chemistry 2, University of Lund, Chemical Centre, S-220 07 Lund, Sweden. *Received July 28, 1983.* Supported by the Swedish National Research Council (NFR).

[1] Abbreviations: CaM, calmodulin; TnC, troponin C; NMR, nuclear magnetic resonance; DEAE, diethylaminoethyl; TFP, trifluoperazine; SDS, sodium dodecyl sulfate; STI, soybean trypsin inhibitor; 2D, two dimensional; EDTA, ethylenediaminetetraacetic acid. Peptide nomenclature: TR_1C, fragment 1–77; TR_2C, fragment 78–148; TM_1, fragment 1–106; TM_2, fragment 107–148 [see Kuznicki et al. (1981), Wall et al. (1981), and Andersson et al. (1983a)].

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et al., 1983) which are thought to be important for the interaction of CaM with its manifold target proteins [for a review, see Cheung (1980) and Klee & Vanaman (1982)]. One calcium-induced hydrophobic site appears to be located in each half of the molecule (Vogel et al., 1983), and the existence of allosteric interactions between these two sites has been reported (Johnson, 1983). Various hydrophobic drugs, like the antipsychotic drug trifluoperazine (TFP), are powerful inhibitors of the potentiating effects of the calmodulin–calcium complex on its target proteins; the corollary to this, that they compete for the same binding sites on CaM's surface, has been widely accepted (Klee & Vanaman, 1982). Thus, in order to gain an understanding of CaM's structure when bound in complexes, several studies have focused on the study of CaM itself (Klevit et al., 1981; Krebs & Carafoli, 1982) or metal ions bound to CaM (Forsén et al., 1980; Thulin et al., 1980; Andersson et al., 1983b) in drug–protein–metal ion complexes.

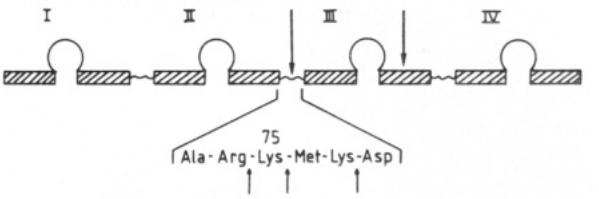
Another question which is of imminent importance to an understanding of the capacity of CaM to regulate such a wide spectrum of different metabolic processes is the order in which the sites of apo-CaM are filled by Ca^{2+}. Although it is generally agreed upon that filling of the sites takes place sequentially, rather than randomly (Klee & Vanaman, 1982; Kilhoffer et al., 1983), many conflicting results have been reported. For TnC, unlike CaM, it is firmly established that domains III and IV are the high-affinity binding sites (McCubbin & Kay, 1980). Instrumental for this assignment were studies using proteolytic fragments that had retained the native structural properties of the intact protein (Leavis et al., 1978). Recent cadmium-113 NMR studies utilizing proteolytic fragments of CaM (Walsh et al., 1977; Drabikowski et al., 1977) provided strong evidence for sites III and IV being the positive cooperative high-affinity binding sites for Ca^{2+} (Andersson et al., 1983a). In addition, our affinity chromatographic studies have provided some insight into the location of the Ca^{2+}-induced hydrophobic domains (Vogel et al., 1983a). Here we report on further ^1H and ^{113}Cd NMR studies of these proteolytic fragments which have shed more light on the location of the high-affinity metal ion binding sites and the hydrophobic drug binding sites. In the course of these studies, we also confirmed that tryptic cleavage of calmodulin in the presence of Ca^{2+} mainly takes place at Lys-77 (Walsh et al., 1977). However, we discovered that considerable hydrolysis of other peptide bonds can occur instead.

Experimental Procedures

Materials. Bovine testis calmodulin and its tryptic and thrombic fragments were prepared as described earlier (Andersson et al., 1983a; Vogel et al., 1983a). All other chemicals used were commercially obtained and were of the highest purity available.

Methods. The purity of CaM and its proteolytic fragments was checked by both SDS gel electrophoresis and agarose gel electrophoresis in the presence and absence of 1 mM EDTA or Ca^{2+}. Amino acid compositions and sequence analysis were performed as described earlier (Vogel et al., 1983a). ^{113}Cd NMR experiments were performed on our home-built 6-T NMR spectrometer [for a description, see Drakenberg et al. (1983)] using routine acquisition parameters described elsewhere (Forsén et al., 1980). ^1H NMR spectra of protein dissolved in D_2O were obtained on a Nicolet 360-WB spectrometer as detailed elsewhere (Andersson et al., 1983b). Two-dimensional (2D) proton correlation spectra were acquired with the standard COSY program of the Nicolet NMCFT software by using a 90°–τ–60°–acquisition pulse sequence with quadrature detection in both dimensions, where

Scheme I



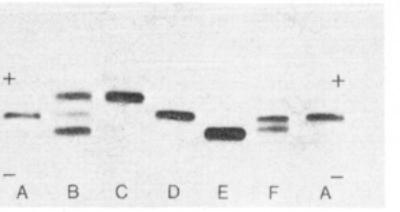


FIGURE 1: Agarose gels (run in the presence of 1 mM EDTA) of CaM and its tryptic fragments: (A) CaM; (B) mixture of $TR_2C_{a,b,c}$; (C) TR_2C_a; (D) TR_2C_b; (E) TR_2C_c; (F) TR_1C. The positions of the cathode and anode are indicated in the figure.

τ was changed in steps of 250 μs corresponding to a spectral width of ±2000 Hz. Routinely, 96–240 scans were accumulated into 512 blocks of 1024 data points. Resolution enhancement of the two-dimensional spectra was achieved by double-exponential multiplication and symmetrization of the spectra around the diagonal (Nagayama et al., 1979). Gated proton decoupling was used to reduce the intensity of the residual HDO resonance during the two-dimensional experiments.

Results

Proteolytic Cleavage of Calmodulin. Scheme I depicts the structure of calmodulin and indicates the reported points of cleavage by thrombin (Arg-106) and trypsin (Lys-77) in the presence of EDTA and Ca^{2+}, respectively (Wall et al., 1981; Walsh et al., 1977). Using amino acid compositions and sequencing studies, we confirmed that thrombic cleavage under these conditions occurs at Arg-106 only (Wall et al., 1981; data not shown). However, agarose gel analysis of the TR_2C fragment of calmodulin[2] consistently showed the presence of three bands (see Figure 1, lane B), although the starting material CaM moved as one band both on agarose gel electrophoresis (see Figure 1, lane A) and on SDS gel electrophoresis. Thus, it appeared that tryptic cleavage of CaM in the presence of Ca^{2+} results in three TR_2C fragments of slightly different charge. Because some differentiation (but no resolution) of these fragments was obtained upon chromatography on a Sephadex G50 (200 × 1.4 cm) column, it appeared that the fragments were only slightly different in size. In order to resolve these three fragments, a degradation mix (Andersson et al., 1983a) was immediately applied to a DEAE-Sephacel column (1 × 34 cm) which was equilibrated in 50 mM sodium acetate, pH 5.5 (adjusted to pH 5.5 with acetic acid). The column was eluted with a combined pH and salt gradient using a buffer containing 50 mM sodium acetate–100 mM acetic acid, pH 3.7 (pH adjusted with HCl). The column profile obtained for this peptide mixture is shown in Figure 2. The peaks that eluted directly from the column contained STI and the trypsin–STI complex; peak D contains undegraded calmodulin as judged by electrophoresis. The

[2] Our standard proteolysis conditions are such that hardly any peptide TR_1C remains in the proteolysis mixture (Andersson et al., 1983a). Thus, TR_2C can be easily purified by gel filtration from leftover undegraded CaM.

Table I: Amino Acid Sequences Determined for Tryptic Calmodulin Carboxy-Terminal Fragments

peptide	amino acid sequence															
			75					80					85			
CaM[a]	A	R	K	M	K	D	T	D	S	E	E	E	I	R	E	A	F
TR_2C_a						D	T	D	S	E	E	E	I/K	R	E	A	F
TR_2C_b				M	K	D	T	D	S	E	E	E	I	R	E	A	F
TR_2C_c			K/L	V/M	K/L	D	T	D	S	E	E	E	I/K	R	E	A	F

[a] From Watterson et al. (1980).

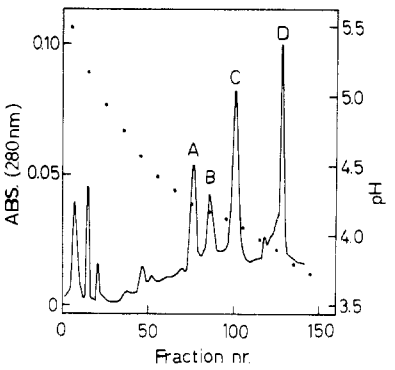


FIGURE 2: Purification of TR_2C peptides on DEAE-Sephacel using a pH gradient (●). Peaks A, B, C, and D correspond to fragments TR_2C_a, TR_2C_b, TR_2C_c, and CaM, respectively. For further explanation, see the text.

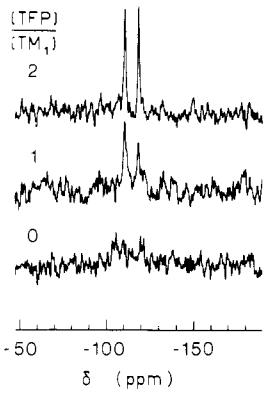


FIGURE 3: Increase of signals in the ^{113}Cd NMR spectrum of Cd^{2+}-saturated TM_1 (0.92 mM, pH 7.0) upon TFP titration (5×10^4 acquisitions). The bottom spectrum cannot be directly compared to that reported earlier (Andersson et al., 1983a) since that was obtained at a somewhat lower temperature.

other peaks labeled A, B, and C were separately dialyzed against double-distilled water and lyophilized. Agarose gel electrophoresis showed that every peak contained a pure TR_2C peptide (see Figure 1, lanes C, D, and E for peaks C, B, and A respectively). Subsequent amino acid analysis (data not shown) and sequencing (Table I) studies provided insight into the nature of the three different peptides. Cleavage does not only occur at Lys-77 but also at Arg-74 and Lys-75, thus giving rise to the three fragments of slightly different charge and length. Since TR_1C also runs as three bands on agarose gel electrophoresis (Figure 1, lane F), it is likely that TR_1C is a mixture of the peptides 1–74, 1–75, and 1–77. However, since the relative intensities of the bands in Figure 1F do not correspond to the yields of the TR_2C peptides, it is likely that further proteolysis of fragments can occur.

Cadmium-113 NMR Studies of Proteolytic Fragments. Titration of CaM with $^{113}Cd^{2+}$ gives rise to two well-resolved signals in the ^{113}Cd NMR spectrum (slow exchange on the NMR time scale) that grow parallel in intensity up to 2 equiv of Cd^{2+} per CaM. Subsequent addition of 2 more equiv does not give rise to any detectable resonance; however, upon addition of further equivalents, a signal for free Cd^{2+} ions is observed (Forsén et al., 1980). The nondetectability of the signals for the third and fourth equivalent of $^{113}Cd^{2+}$ has been attributed to chemical exchange between free and protein-bound Cd^{2+} ions (Forsén et al., 1980). Since monovalent cations like Na^+ and K^+ can bind to the protein (Delville et al., 1980; Haiech et al., 1981), it appeared worthwhile to investigate the effect of salts on the binding of metal ions. However, titration experiments performed in the presence of 0.15 M $KClO_4$ gave results identical with those discussed above.

We have recently shown that addition of 2 equiv of $^{113}Cd^{2+}$ to peptide TR_2C (in fact a mixture of the three TR_2C fragments) gives rise to the same two well-resolved resonances as for CaM (Andersson et al., 1983a). No resonance is observed if 2 equiv of $^{113}Cd^{2+}$ is added to fragment TR_1C. Subsequent addition of Ca^{2+} liberates the bound $^{113}Cd^{2+}$ ions which results in a broad resonance at −12 ppm for Cd^{2+} (spectra included in the supplementary material; see paragraph at end of paper regarding supplementary material). Thus, the two calcium binding sites of CaM that exhibit the intermediate to fast chemical exchange are located in this fragment, consistent with the observation that the two slow exchanging sites are located in fragment TR_2C. In further experiments, we have attempted to visualize the two Cd^{2+} ions bound to TR_1C by trying to reduce the metal ion exchange rate by lowering the temperature. However, a reduction from 23 to 2 °C had no effect on the ^{113} Cd NMR spectrum of TR_1C. Similar low-temperature spectra were obtained for CaM, TR_2C, TM_1, and TM_2. We found that the broad signals observed for Cd^{2+} bound to TM_1 and TM_2 (Andersson et al., 1983a) sharpened somewhat. In addition, all observable protein-bound $^{113}Cd^{2+}$ resonances showed small downfield shifts when the temperature was lowered to 2 °C (varying between 1 and 4.5 ppm). These effects are comparable to those observed for the $^{113}Cd^{2+}$ NMR reference signal [0.1 M $Cd(ClO_4)_2$] which itself shifts 3.5 ppm downfield between 23 and 2 °C.

Effect of Trifluoperazine on the ^{113}Cd NMR Fragment Spectra. Addition of hydrophobic drugs to CaM results in large changes in the ^{113}Cd NMR spectra (Forsén et al., 1980; Andersson et al., 1983b). The two most pronounced effects that occur upon binding of 2 equiv of TFP are the following: (1) an upfield shift for the two slow exchange signals; (2) a drastic reduction of the exchange rate for the fast to intermediate exchanging $^{113}Cd^{2+}$ ions, so that two additional sharp resonances (<50 Hz) appear in the spectrum.[3] Figure 3 shows the effect of the addition of TFP on the ^{113}Cd NMR spectrum of TM_1 (1–106). Two sharp resonances appear, indicating that the drug binding site responsible for the decrease in the metal ion exchange rate of the two weak metal ion binding sites is

[3] Since the on rate of the metal ion for all four Ca^{2+} binding sites is thought to be diffusion limited, a decreasing off rate (approximately equal to the metal ion exchange rate as detected by NMR) indicates an increase in the binding constant. Also, ^{43}Ca NMR studies have provided evidence for a tighter binding of metal ions in the presence of TFP (Thulin et al., 1980).

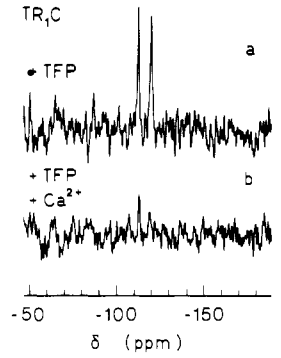


FIGURE 4: ^{113}Cd NMR spectra of Cd^{2+}-saturated TR_1C (0.97 mM, pH 7.5) after addition of (a) 2 equiv of TFP and (b) 3 equiv of TFP and 2 equiv of Ca^{2+} (5×10^4 acquisitions).

Table II: Chemical Shifts Measured for the Protein-Bound ^{113}Cd Signals in the Absence and Presence of Trifluoperazine

protein	[TFP]: [protein]	chemical shift (ppm) A	C	D	B
CaM	0	−87.5	n.o.[a]	n.o.	−114.2
	2.7	−99.1	−112.9	−115.2	−118.1
TR_2C	0	−87.5			−114.2
	3.0	−100.0			−118.0
TR_1C	0		n.o.	n.o.	
	3.0		−112.0	−119.5	
TM_1	0		n.o.	n.o.	−109[b]
	3.0		−111.5	−119.0	n.o.
TM_2	0	−95[b]			
	2.0	−95[b]			

[a] n.o. = not observable due to exchange broadening. [b] Broad resonance (>200 Hz).

located in this peptide and is functioning as in the native protein. The same two resonances are observed with peptide TR_1C after addition of TFP (see Figure 4a). When TFP was added to a sample containing $^{113}Cd^{2+}$-loaded TR_2C, the two sharp Cd^{2+} signals shifted as for CaM (see Table II). Also, the broadening observed for the most downfield resonance in the midpoint of the titration of CaM [see Figure 2D in Forsén et al. (1980)] was observed here. It is further noteworthy that when one adds up the spectra for TFP-saturated TR_1C and TR_2C a spectrum closely resembling that of TFP-saturated CaM is obtained (compare Table II). Significantly, addition of TFP to fragment TM_2 (107–148) did not cause a change in the ^{113}Cd NMR spectrum. We also investigated competition between Ca^{2+} and Cd^{2+} in these TFP-saturated peptides (see, for example, Figure 4). In all instances where ^{113}Cd NMR signals with chemical shifts between −80 and −120 ppm were observed, these signals disappeared after the addition of an excess of Ca^{2+} ions, and a signal with a chemical shift close to that of free Cd^{2+} ions appeared.

^1H NMR Structural Comparison between CaM and Fragments. Figures 5 and 6 compare the aromatic and the upfield-shifted methyl group regions of the 360-MHz ^1H NMR spectra of apo- and calcium-loaded CaM, TR_1C, and TR_2C as well as the difference spectrum CaM − (TR_1C + TR_2C) which was obtained by computer subtraction. Extensive studies by other groups have provided tentative assignments of various resonances to specific residues for apo-CaM as well as Ca^{2+}-CaM (Seamon, 1980; Krebs & Carafoli, 1982; Ikura et al., 1983a,b). A listing of the assignments of the most important resonances is shown in Table III. Proton resonances that are normally most sensitive to changes in the protein's structure are those that are shifted by ring current effects induced by proximal aromatic groups. Such resolved

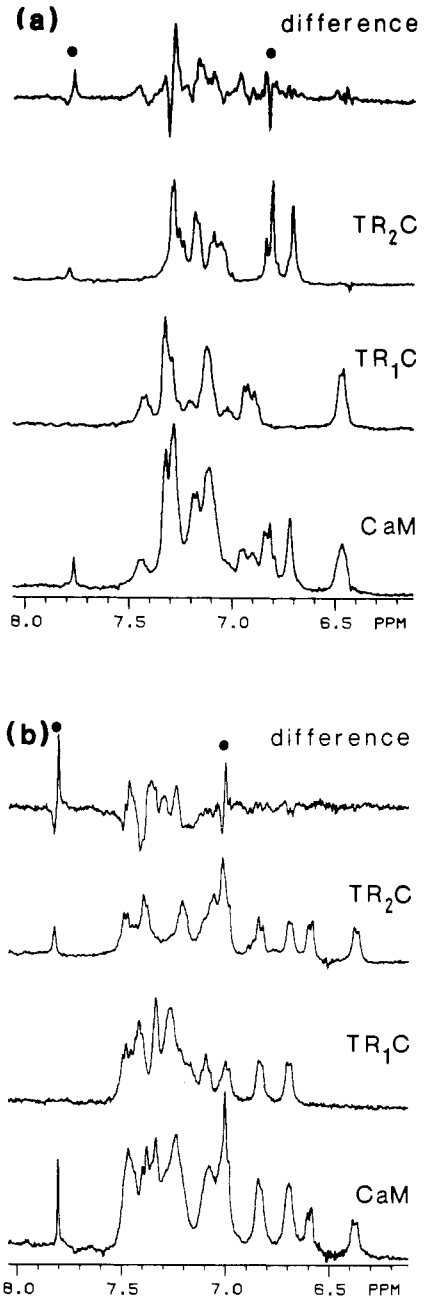


FIGURE 5: Aromatic region of the ^1H NMR spectra of TR_1C, TR_2C (both 1.5 mM, pH 7.5), and CaM (2.0 mM, pH 7.5) and the difference spectra of CaM − (TR_1C + TR_2C): (a) apo forms; (b) Ca^{2+}-saturated forms. The solid dots indicate the positions of the His-107 resonances, which are very sensitive to small changes in pH and hence show up in the difference spectra (500 acquisitions).

resonances are found between 6.3 and 6.9 ppm and −0.2 and 0.6 ppm in the spectra. Visual inspection reveals the great similarity for both the apo- and the calcium-saturated CaM in these regions of the spectra (Figures 5 and 6). For example, the two most highfield-shifted phenylalanine resonances in the TR_1C fragment as well as the most highfield phenylalanine and the four tyrosine resonances in the TR_2C fragment all have the same shift as in calmodulin itself and consequently disappear in the difference spectrum (Figure 5a). This indicates that the two halves of the CaM molecule have the same conformation whether they are covalently attached to each other as in calmodulin or physically separated as in the fragments. The same holds for the apoprotein as well as for the Ca^{2+}-loaded protein. A similar comparison of the ^1H NMR spectra from the two thrombic fragments with that of intact calmodulin shows that these fragments do not have the

Table III: Assignments of Proton Resonances of Calmodulin and Its Fragments[a]

residue	proton	chemical shift (ppm)[d] Ca^{2+} free	chemical shift (ppm)[d] Ca^{2+} loaded	proteolytic fragment	conformational exchange rate
His-107	δ	7.85[b]	7.79[b]	TR_2C	slow
	ϵ	6.82[b]	7.00[b]	TR_2C	
Tyr-99	δ	6.86	6.82	TR_2C	slow
	ϵ	7.14	6.98	TR_2C	
Tyr-138	δ	6.75	6.36	TR_2C	slow
	ϵ	6.75	6.58	TR_2C	
Tml-115[c]	ϵ	3.13	3.11	TR_2C	slow
Phe-A	δ	6.47	6.64	TR_1C	fast
Phe-B	δ	6.47	6.78	TR_1C	fast
Val-A	γ	0.52		TR_1C	
Val-B	γ	0.43		TR_2C	slow
Ile-A	γ	−0.14		TR_1C	fast
	δ	0.33		TR_1C	fast

[a] Assignments from Seamon (1980), Krebs & Carafoli (1982), and Ikura et al. (1983a,b). [b] pH dependent. [c] Tml is trimethyllysine. [d] Our observation for both fragments and CaM.

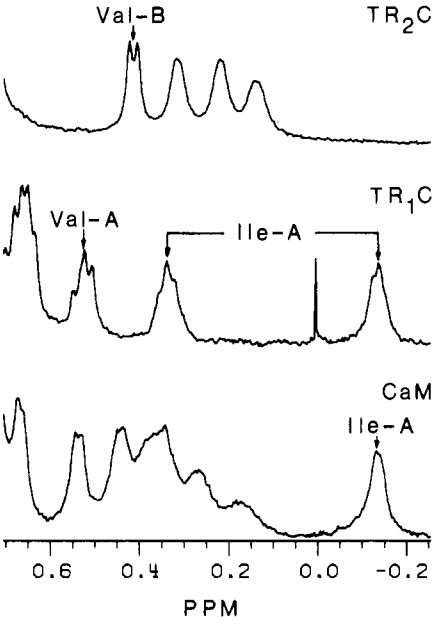


FIGURE 6: Most upfield part of the ^1H NMR spectra of TR_1C, TR_2C, and CaM in the apo form. (See Figure 7 for other conditions. Spectra of the Ca^{2+} form are included in the supplementary material.)

same conformation as the intact protein (data not shown).

A further test for the similarity of the structure of the fragments as compared to the intact protein can be obtained from 2D NMR. In this way, not only the similarity of individual chemical shifts but also correlation signals from resonances connected by spin couplings can be checked. Figure 7 shows the contour plots for the COSY 2D NMR spectra of calmodulin and its two tryptic fragments. Such a contour plot contains the normal one-dimensional NMR spectrum (viewed from above) along the main diagonal; spin–spin couplings are revealed as off-diagonal peaks connecting the two coupled resonances on the diagonal. It is obvious from Figure 7 that all correlation signals observed in the calmodulin spectrum can also be found in either of the spectra from the fragments. Some extra correlation signals can be observed in the 2D spectra of the fragments, which were not detectable in the 2D spectrum from calmodulin, most probably caused by the fact that the resonances for the lower molecular weight fragments tend to be somewhat sharper and thus give rise to larger cross-peaks.

From a comparison of the spectra obtained for the two tryptic fragments and CaM (Figures 5 and 6), one can decide what resonance in the ^1H NMR spectrum of CaM resides in what half of the protein. We have listed in Table III in which fragment the corresponding resonances were found. As expected, the resonances for tyrosines, histidine-107, and trimethyllysine-115 appear in fragment TR_2C. However, the tentative assignment of Phe-A, -B, and -Z to residues 89, 92, and 141 (Ikura et al., 1983a,b) appears at error since Phe-A and -B are found in the TR_1C fragment. Moreover, Val-A and Ile-A, that are known to be located close to Phe-A and -B, are also in the amino-terminal half of CaM, whereas Val-B is part of the carboxy-terminal half.

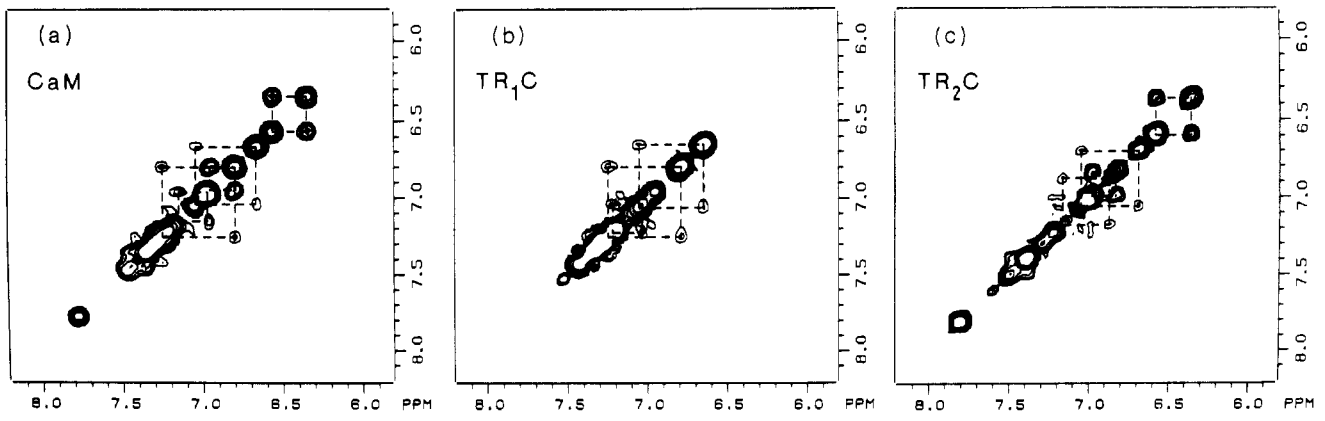


FIGURE 7: Aromatic part of the 2D COSY ^1H NMR spectra of CaM and its tryptic fragments: (a) 2.0 mM CaM, 8.0 mM Ca^{2+}, pH 7.5, 240 scans; (b) 1.8 mM TR_1C, 3.6 mM Ca^{2+}, pH 7.6, 96 scans; (c) 1.7 mM TR_2C, 3.5 mM Ca^{2+}, pH 7.5, 128 scans. For other conditions see Experimental Procedures.

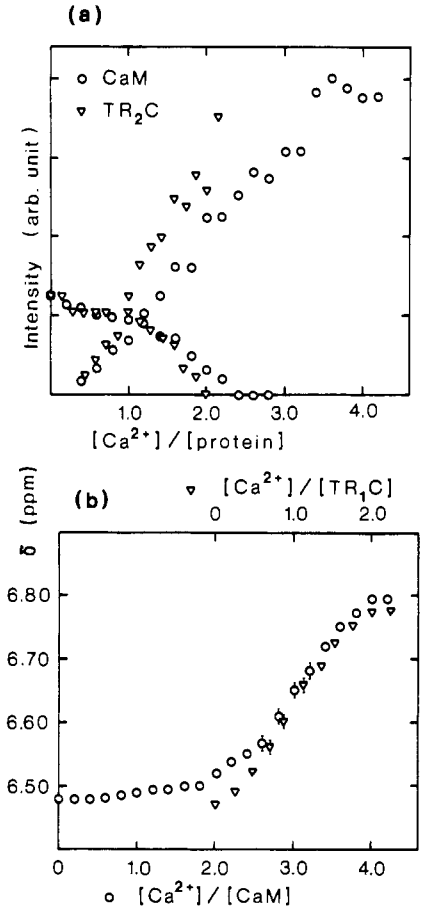


FIGURE 8: Changes in the ^1H NMR spectra of CaM and its fragments upon Ca^{2+} addition: (a) intensity of the His-107 resonance in the spectrum of TR_2C (1.5 mM, pH 7.5) or CaM; (b) chemical shift of one upfield-shifted phenylalanine in the spectrum of TR_1C (1.5 mM, pH 7.5) or CaM (2.0 mM, pH 7.5).

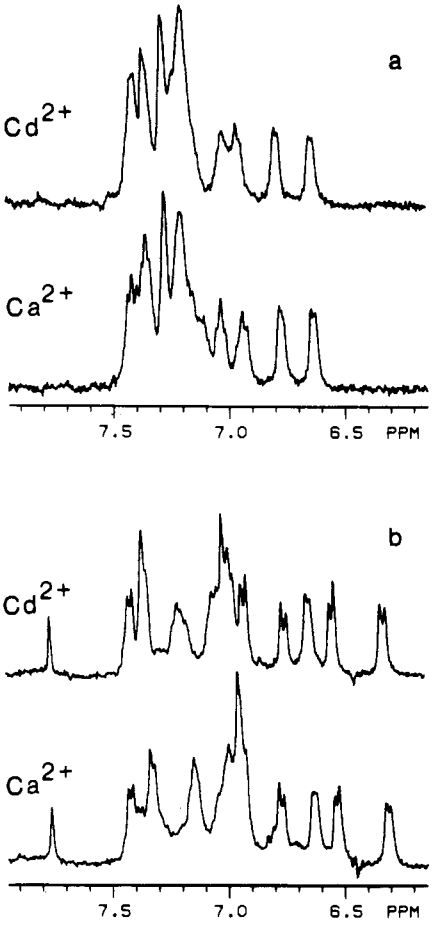


FIGURE 9: Aromatic regions of the ^1H NMR spectra of the tryptic fragments. The Ca^{2+}- and the Cd^{2+}-saturated forms are shown: (a) 1.5 mM TR_1C, pH 7.5; (b) 1.5 mM TR_2C, pH 7.5 (500 scans). For spectra of the apo fragments, see Figure 5.

Ca^{2+}-Induced Conformational Changes in the Tryptic Fragments. Figure 8a shows the intensities of some resonances in the ^1H NMR spectra of CaM and TR_2C as a function of Ca^{2+} saturation. Three resonances from aromatic amino acids can easily be followed as they are well resolved from other resonances in the aromatic region for both the intact protein and the fragments. These resonances are His-107 at 7.85 ppm in the metal-free state and at 7.79 ppm in the Ca^{2+}-loaded state and Tyr-138 at 6.36 ppm in the calcium-saturated state. Since the conformational change due to Ca^{2+} binding in both CaM and TR_2C is in slow exchange (on the proton NMR time scale), a decrease in the intensity of the signals from the apoproteins and an increase in the intensities of the signals from the calcium-saturated proteins can be observed [see Seamon (1980) and Ikura et al. (1983b)]. These changes are observed to occur in an almost identical way for CaM and TR_2C as can be seen from Figure 8a. As expected, the resonances from trimethyllysine-115 (3.13 ppm in the apoprotein and 3.11 ppm when calcium saturated) and tyrosine-138 (and Tyr-99) go through a similar variation as the His-107 resonance (see Table III).

Figure 8b shows the chemical shifts of one of the two phenylalanine resonances in the ^1H NMR spectra of CaM and TR_1C as a function of added calcium ions. In the spectrum of TR_1C, these particular resonances are well resolved from other aromatic resonances, while in the spectrum of CaM, overlap with other resonances takes place during the latter half of the Ca^{2+} titration. For CaM, no alteration of the chemical shifts of these signals could be observed up to a Ca^{2+}:CaM ratio of 2:1.[4] After this ratio, a continuous change of the chemical shifts can be observed until the final shifts of 6.64 and 6.78 ppm are reached for a Ca^{2+}:CaM ratio of 4:1 ($CaM–Ca_4$) (Table III). This change is almost identical with the variation in the chemical shifts observed for TR_1C when the Ca^{2+}:TR_1C ratio is varied from 0:1 to 2:1 (see Figure 8b).

Comparison of the Effects of Ca^{2+} and Cd^{2+} on the ^1H NMR Spectra of the Tryptic Fragments. Figure 9 shows the aromatic part of the ^1H NMR spectra of Ca^{2+}- and Cd^{2+}-saturated TR_1C and TR_2C fragments. In the ^1H NMR spectrum of TR_1C, the two highfield-shifted phenylalanine resonances (Phe-A and -B) at 6.47 ppm as well as two others (Phe-C and -D) around 6.9 ppm shift downfield and split into two resonances as the metal ion content is increased. Although the same sequence of events is observed during the calcium as well as the cadmium titration, slight differences of the chemical shifts are measured for the Phe-C and -D resonances (6.94 and 7.04 ppm in $TR_1C–Ca_2$ and 6.95 and 7.02 ppm in $TR_2C–Cd_2$). Also in the region of the unshifted phenylalanine resonances, particularly at 7.5 ppm, some minor differences can be observed. A broadening of the highfield-shifted phenylalanine signals can be seen in the course of the titration. The effect is most pronounced in the case of calcium binding.

[4] Ikura et al. (1983a,b) indicate that Phe-A is affected by both the slow and the fast exchange process, and thus changes during titration between zero and four Ca^{2+} ions, and that Phe-B is affected by the slow exchange process only. However, our calcium titrations on CaM as well as its tryptic fragments do not show this behavior. In our hands, both resonances are only affected by the fast exchange process, which occurs on binding of the third and fourth Ca^{2+} ions to CaM.

This broadening can be used to calculate the lifetime of the conformation induced by calcium or cadmium ion binding to TR_1C. Using $\tau = 2\Delta\nu_{1/2}/(\pi\delta\nu^2)$, where $\Delta\nu_{1/2}$ is the maximum broadening of the line and $\delta\nu$ the chemical shift difference between the two exchanging species, we have calculated the lifetime, τ, to be ca. 0.4 and ca. 0.8 ms for TR_1C–Cd_2 and TR_1C–Ca_2, respectively. In other regions of the proton NMR spectrum of TR_1C, similar effects can be found as in the aromatic region. For example, the highfield Ile-A resonance at −0.14 ppm broadens when Ca^{2+} or Cd^{2+} is added and shifts downfield. However, it should be indicated here that the proton NMR spectra of the Ca^{2+}- and Cd^{2+}-loaded TR_1C are not exactly identical in the methyl region but display small differences in chemical shifts.

As can be seen in Figure 9, the Ca^{2+}- and Cd^{2+}-saturated fragment TR_2C's give rise to very similar proton NMR spectra. However, as for the TR_1C fragment, there are some minor differences; for example, the Tyr-99 resonances appear at 6.82 and 6.98 ppm and at 6.79 and 6.96 ppm in the Ca^{2+}- and Cd^{2+}-loaded forms, respectively. Furthermore, a broadening of the signals in the proton NMR spectra during the cadmium titration is observed, which was not seen in the calcium titration. This broadening corresponds to a lifetime of the TR_2C–Cd_2 complex of ca. 20 ms. The lack of broadening in the calcium titration indicates that the lifetime of the TR_2C–Ca_2 complex is long, $\tau > 0.5$ s.

Discussion

Proteolytic Fragmentation. Since Ca^{2+} binding to CaM causes large conformational changes, proteolytic studies of CaM performed in the presence of either EDTA or Ca^{2+} have given widely different results. Walsh et al. (1977) have provided evidence that proteolytic cleavage of CaM in the presence of Ca^{2+} occurs at Lys-77. The results presented in this paper indicate that indeed ~55% of the hydrolysis products formed is the peptide 78–148. However, a considerable degree of cleavage at Arg-74 (~25%) and Lys-75 (~20%) was observed as well, as the determined amino acid compositions and amino acid sequences (Table I) indicate. Since not only TR_2C but also fragment TR_1C runs as the three bands of different charge, it is clear that proteolytic attack can occur at either one of these three positions, rather than that the main product 78–148 arises from further degradation of the 75–148 or the 76–148 peptide. Thus, this implies surface location and considerable flexibility for these three residues in the presence of Ca^{2+}. These results are in excellent agreement with chemical modification studies showing that carboxymethylation of the methionine residues 71, 72, and 76 was facilitated by the presence of Ca^{2+} (Walsh & Stevens, 1978). Since this modification prevents the productive interaction of CaM with cyclic phosphodiesterase, it appears that the Ca^{2+}-exposed flexible hinge region connecting CaM's D and E helices as well as helix D plays an important role in the interaction of CaM with its target proteins.

Cadmium-113 NMR Studies of Proteolytic Fragments of Calmodulin. In order for ^{113}Cd NMR of proteolytic fragments of calmodulin to be a valid approach for determining the calcium binding properties of the intact protein, the following three conditions have to be fulfilled: (1) the conformation of the fragments has to closely resemble that of the intact protein; (2) the conformational changes induced by binding of metal ions have to be the same for the fragments and CaM; (3) Cd^{2+} has to act similarly to Ca^{2+}. These three conditions are discussed in the following sections.

(1) Our ^1H NMR data presented in Figures 5–7 provide strong evidence for the idea that the NMR spectrum of intact CaM is closely resembled by the sum of the spectra of its two tryptic fragments. Particularly the observation that all the ring current shifted resonances, which are the most sensitive ^1H NMR parameters of a protein's folding, appear at identical chemical shifts implies that both halves of calmodulin must be independently folded. Although binding of Ca^{2+} causes conformational changes resulting in large changes in the pattern of ring current shifted resonances, the agreement between the sum of the tryptic spectra and that of CaM, for both the apo- and the Ca^{2+}-loaded forms of the protein, is very good. Studies using a series of other spectroscopic methods are in good agreement with our ^1H NMR data since they also indicate the structural similarity between the conformations of the two independent halves of the protein and the intact CaM (Drabikowski et al., 1982). Thus results obtained with the tryptic (but not the thrombic) fragments can be extrapolated to intact CaM.

(2) With regard to the second question, our ^1H NMR data, as presented in Figure 8, for example, clearly indicate that binding of Ca^{2+} causes the same sequence of events when binding to CaM or to its tryptic fragments (see also the section below on the sequence of filling). Titration studies using other spectroscopic techniques also provided evidence for the fact that the same conformational changes are obtained for the tryptic fragments and intact CaM (Drabikowski et al., 1982).

(3) The final requirement (that Cd^{2+} has to act similarly to Ca^{2+}) has been studied in various ways. Studies in our laboratory on proteins homologous to CaM have shown that Cd^{2+} is a reliable replacement for Ca^{2+} [see Vogel et al. (1983b) and Forsén et al. (1983)]. Since both Cd^{2+} and Ca^{2+} induce the same change in electrophoretic mobility (Andersson et al., 1983a), the conformations of Cd^{2+}- and Ca^{2+}-saturated CaM and fragments appear to be similar. The most demanding test for Cd^{2+} as a reliable Ca^{2+} substitution probe is provided, however, by following the changes in the ^1H NMR spectra for both Ca^{2+} and Cd^{2+} in titration experiments. For intact CaM, we and Klevitt (1981) have shown that addition of each metal ion causes almost identical changes in the ^1H NMR spectra; only minor differences are observed in the metal ion saturated CaM (Forsén et al., 1983). Figure 9 compares the spectra for the tryptic fragments obtained in the presence of Ca^{2+} or Cd^{2+}. The spectra are very similar; only minor differences can be detected. During the titration from 0 to 2 equiv of Ca^{2+} and Cd^{2+}, the same sequence of events take place (i.e., resonances shift the same directions and approximately at the same rates in both titrations). Thus, on the whole, these data are all in support of the idea that Cd^{2+} is a reliable substitute for Ca^{2+}, since it induces conformational changes in the same order and to the same extent as calcium.

Assignment of Strong and Weak Cation Binding Sites. The ^{113}Cd NMR spectrum of TR_2C resembles CaM–Cd_2 (Andersson et al., 1983a) whereas the ^{113}Cd NMR spectrum of Cd^{2+}-loaded TR_1C shows no resonances. This resembles what happens when the third and fourth equivalent of Cd^{2+} is added to CaM–Cd_2 to give CaM–Cd_4. Thus, these data indicate that domains III and IV are the high-affinity metal ion binding sites and domains I and II are the low-affinity sites. Further evidence for this sequence of filling is provided by the ^1H NMR studies of the proteolytic fragments. In ^1H NMR studies of intact CaM, resonances could be divided in three groups depending on their Ca^{2+} titration behavior: group I, slow conformational exchange on the ^1H NMR time scale (one signal increases and concomitantly another decreases); group II, fast exchange (a signal shifts gradually from one chemical shift position to another); group III, resonances affected by

both processes (Seamon, 1980; Ikura et al., 1983b). The results presented here (see Figure 8 and Table III) indicate that all the resonances sensing the slow exchange processes are located in the carboxy-terminal fragment, whereas the resonances associated with the fast exchange process are located in the amino-terminal fragment. Thus, these ^1H NMR studies on the proteolytic fragments also show that the carboxy-terminal half contains the two high-affinity sites, where Ca^{2+} binding gives rise to slowly exchanging conformations, and that domains I and II are the low affinity sites. Although the same pattern had been previously inferred from ^1H NMR studies of intact CaM (Seamon, 1980; Ikura et al., 1983b), one can never be sure that binding to half of the molecule does not cause large conformational changes that are transmitted to the other side of the molecule. However, since the results with the proteolytic fragments and CaM reported here are similar, we can conclude that the majority of the conformational rearrangements detected in the ^1H NMR spectra must be localized to the half of the protein containing the calcium binding sites involved in Ca^{2+} binding.

As discussed elsewhere (Andersson et al., 1983a), other researchers have suggested other schemes for the ordered filling of CaM's metal ion binding sites. However, the majority of these studies used trivalent lanthanides (Kilhoffer et al., 1983; Wang et al., 1982; Wallace et al., 1982; Krebs & Carafoli, 1982) or monovalent cations (Delville et al., 1980; Haiech et al., 1981) to probe the calcium binding sites of CaM. Although lanthanides do support CaM activity (Wallace et al., 1982; Klee & Vanaman, 1982), no evidence has been presented to date that they have the same sequence of filling as Ca^{2+}. In fact, the conformational changes observed upon titration of CaM with La^{3+} that can be followed by ^1H NMR indicate a different sequence of filling for this cation than for Ca^{2+} and Cd^{2+} (W. Niemczura, unpublished results). Also, the studies using monovalent cations rely on the assumption that these cations and Ca^{2+} compete for the same metal ion binding sites (Delville et al., 1980; Haiech et al., 1981). This assumption is not necessarily correct. β-Parvalbumins, for example, contain a monovalent cation binding site close to one of the Ca^{2+}–EF hand sites, whose metal ion binding properties are strongly influenced by occupation of the EF hand site (Cavé et al., 1982). Similar sites could exist in CaM or TnC and could explain the reported observations.

It should be noted that the assignment of domains III and IV as the strong binding sites would make calmodulin in this respect similar to skeletal TnC (Leavis et al., 1978; Sin et al., 1978) and cardiac TnC (Hincke et al., 1981; Teleman et al., 1983). This order of binding has also been predicted on the basis of the amino acid similarities between TnC and CaM (Reid & Hodges, 1980; Klee & Vanaman, 1982).

Interaction of Calmodulin and Drugs. Since binding of TFP to TR_1C and TR_2C causes specific changes in the ^{113}Cd NMR spectra of these fragments (Figures 3 and 4, Table II) that are virtually indistinguishable from those observed upon titration of intact CaM with TFP (Forsén et al., 1980), it appears that the two binding sites for the TFP molecules are still largely intact. Thus, one such calcium-induced binding site is located in the amino-terminal half of the protein and the other in the carboxy-terminal half of the protein (Vogel et al., 1983). The location of the latter site is most probably not solely on the carboxy-terminal helix of CaM as had been suggested (Klevit et al., 1981), since no changes in the ^{113}Cd NMR spectrum of fragment TM_2 were observed upon TFP addition. In fact, evidence has been provided for its location in the fragment 77–124 (Head et al., 1982), although location in the stretch 77–106 appears less likely (Vogel et al., 1983a). From a comparison of the changes in the ^{113}Cd NMR spectra of the tryptic fragments (Figures 3 and 4) and intact CaM [see Figure 2 in Forsén et al. (1980)], one can in fact conclude that the TFP binding site that is located on the TR_2C fragment has a higher affinity for this drug than the binding site located in the amino-terminal half: upon binding of the first TFP molecule to CaM, the changes in the CaM spectrum resemble those observed for TR_2C, whereas the changes observed with TR_1C resemble mostly those observed upon binding of the second TFP molecule to CaM.

Calmodulin Action. Although all the studies discussed above indicate that the two tryptic fragments bear a remarkable resemblance to intact CaM, it would be too simple to suggest that CaM is nothing more than the sum of these two parts. The fact that higher levels of TFP than normally needed for CaM were required in this study to saturated the TR_1C fragment indicates a weaker binding for this hydrophobic drug. This may be caused by the fact that the allosteric interactions between the two drug binding sites (Johnson, 1983) are lost in the fragments. The fact that such interactions exist, plus the existence of a group of proton resonances sensitive to the filling of all four Ca^{2+} binding sites (Seamon, 1980; Ikura, 1983a), indicates that the two independently folded halves of calmodulin do interact with one another. Another point of interest is that proteolytic fragments in general have been disappointingly poor in imitating CaM's effects on the activity of its target enzymes and proteins (Walsh et al., 1977; Kuznicki et al., 1981; Wall et al., 1981; Schreiber et al., 1981). These observations appear to indicate that binding of both Ca^{2+}-exposed "drug" binding domains to the majority of the target enzymes is a necessity to bring about the changes in activity. Klee & Vanaman (1982) pointed out that it is unfortunate that in most of the fragment studies only activation and not the binding of fragments has been determined. If binding of both interaction domains is a requirement for activation, binding of a fragment, with only one interaction site, need not be accompanied by activation. Thus, as a working hypothesis for further experiments, it seems that calmodulin bears a closer resemblance to skeletal TnC than previously thought (Kilhoffer et al., 1983). Positive cooperative binding of the first two Ca^{2+} ions to domains III and IV induces the exposure of the carboxy-terminal interaction site; this is thought to be sufficient to establish binding between CaM and a target enzyme but not necessarily activation. Likewise, the interaction between TnC and the other troponin subunits is fully dependent on the occupancy of its two high-affinity metal binding sites by Ca^{2+} or Mg^{2+} (Cox et al., 1981). As a second step, saturation of CaM's weaker binding sites I and II would expose the second interaction site,[5] which would confer the regulatory action on the activity. In parallel, for TnC, occupation of these sites is considered to be the main regulatory mechanism for muscle contraction (Robertson et al., 1981). It is clear that this mode of CaM's functioning requires further study. However, it does appear to provide a rationale for the intracellular appearance of carboxy-terminal fragments of CaM (Schreiber et al., 1981). These can act as regulators since they can bind to target enzymes and thus block CaM's

[5] This model would fit with the fact that mostly three or four bound Ca^{2+} ions per CaM are required for activation. Certain target enzymes require, however, only two bound Ca^{2+} ions (Klee & Vanaman, 1982). For these, it appears either that binding of the second activation domain is not necessary (a notion not supported by the results obtained by the activation experiments with the proteolytic fragments) or that such binding can take place, without Ca^{2+} binding to sites I and II.

activating interactions, as was recently demonstrated (Newton & Klee, 1983).

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Supplementary Material Available

Two figures showing ^{113}Cd NMR spectra of Cd^{2+} binding to TR_1C as well as a comparison of the upfield-shifted methyl region ^1H NMR spectra of Ca^{2+}-saturated CaM, TR_1C, and TR_2C (2 pages). Ordering information is given on any current masthead page.

Registry No. TFP, 117-89-5; Ca, 7440-70-2; Cd, 7440-43-9; ^{113}Cd, 14336-66-4.

References

Andersson, A., Forsén, S., Thulin, E., & Vogel, H. J. (1983a) *Biochemistry 22*, 2309–2313.

Andersson, A., Drakenberg, T., Thulin, E., & Forsén, S. (1983b) *Eur. J. Biochem. 134*, 459–465.

Cavé, A., Saint-Yves, A., Parello, J., Swärd, M., Thulin, E., & Lindman, B. (1982) *Mol. Cell. Biochem. 44*, 161–172.

Cheung, W. Y. (1980) *Science (Washington, D.C.) 207*, 19–26.

Cox, J. A., Comte, M., & Stein, E. A. (1981) *Biochem. J. 195*, 205–211.

Delville, A., Grandjean, J., Laszlo, P., Gerday, C., Brzeska, H., & Drabikowski, W. (1980) *Eur. J. Biochem. 109*, 515–522.

Drabikowski, W., Kuznicki, J., & Grabarek, Z. (1977) *Biochim. Biophys. Acta 485*, 124–133.

Drabikowski, W., Brzeska, H., & Vanyaminov, S. Y. (1982) *J. Biol. Chem. 257*, 11584–11590.

Drakenberg, T., Forsén, S., & Lilja, H. (1983) *J. Magn. Reson. 53*, 412–423.

Forsén, S., Thulin, E., Drakenberg, T., Krebs, J., & Seamon, K. (1980) *FEBS Lett. 117*, 189–194.

Forsén, S., Andersson, A., Drakenberg, T., Teleman, O., Thulin, E., & Vogel, H. J. (1983) in *Calcium-Binding Proteins, 1983* (de Bernard, B., et al., Eds.) pp 121–132, Elsevier, New York.

Haiech, J., Klee, C. B., & Demaille, J. G. (1981) *Biochemistry 20*, 3890–3897.

Head, J. F., Masure, H. R., & Kaminer, B. (1982) *FEBS Lett. 137*, 71–74.

Hincke, M. T., Sykes, B. D., & Kay, C. M. (1981) *Biochemistry 20*, 4185–4193.

Ikura, M., Hiraoki, T., Hikichi, K., Mikuni, T., Yazawa, M., & Yagi, K. (1983a) *Biochemistry 22*, 2568–2572.

Ikura, M., Hiraoki, T., Hikichi, K., Mikuni, T., Yazawa, M., & Yagi, K. (1983b) *Biochemistry 22*, 2573–2579.

Johnson, J. D. (1983) *Biochem. Biophys. Res. Commun. 112*, 787–793.

Kilhoffer, M. C., Haiech, J., & Demaille, J. G. (1983) *Mol. Cell. Biochem. 51*, 33–45.

Klee, C. B., & Vanaman, T. C. (1982) *Adv. Protein Chem. 35*, 214–321.

Klevit, R. E. (1981) Ph.D. Thesis, University of Oxford, Oxford, England.

Klevit, R. E., Levine, A., & Williams, R. J. P. (1981) *FEBS Lett. 123*, 25–29.

Krebs, J., & Carafoli, E. (1982) *Eur. J. Biochem. 124*, 619–627.

Kretsinger, R. H. (1976) *Annu. Rev. Biochem. 45*, 239–266.

Kuznicki, J., Grabarek, Z., Brzeska, H., Drabikowski, W., & Cohen, P. (1981) *FEBS Lett. 130*, 141–145.

LaPorte, D. C., Wierman, B. M., & Storm, D. R. (1980) *Biochemistry 19*, 3814–3819.

Leavis, P. C., Rosenfeld, S. S., Gergely, J., Grabarek, Z., & Drabikowski, W. (1978) *J. Biol. Chem. 253*, 5452–5459.

McCubbin, W. D., & Kay, C. M. (1980) *Acc. Chem. Res. 13*, 185–192.

Nagayama, K., Wütrich, K., & Ernst, R. R. (1979) *Biochem. Biophys. Res. Commun. 30*, 305–311.

Newton, D. L., & Klee, C. B. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2622.

Robertson, S. P., Johnson, J. D., & Potter, J. D. (1981) *Biophys. J. 34*, 559–569.

Schreiber, W. E., Sasagawa, T., Titani, K., Wade, R. D., Malencik, D., & Fischer, E. H. (1981) *Biochemistry 20*, 5239–5245.

Seamon, K. B. (1980) *Biochemistry 19*, 207–215.

Sin, I. L., Fernandes, R., & Mercola, D. (1978) *Biochem. Biophys. Res. Commun. 82*, 1132–1139.

Tanaka, T., & Hidaka, H. (1980) *J. Biol. Chem. 255*, 11078–11080.

Teleman, O., Drakenberg, T., Forsén, S., & Thulin, E. (1983) *Eur. J. Biochem. 134*, 453–457.

Thulin, E., Forsén, S., Drakenberg, T., Andersson, T., Seamon, K. B., & Krebs, J. (1980) in *Calcium-Binding Proteins: Structure and Function* (Siegel, F. L., et al., Eds.) pp 243–244, Elsevier, New York.

Vogel, H. J., Lindahl, L., & Thulin, E. (1983a) *FEBS Lett. 157*, 241–246.

Vogel, H. J., Drakenberg, T., & Forsén, S. (1983b) in *NMR of Newly Accessible Nuclei* (Laszlo, P., Ed.) Vol. 1, pp 157–192, Academic Press, New York.

Wall, C. M., Grand, R. J., & Perry, S. V. (1981) *Biochem. J. 195*, 307–316.

Wallace, R. W., Tallant, E. A., Docktor, M. E., & Cheung, W. Y. (1982) *J. Biol. Chem. 257*, 1845–1851.

Walsh, M., & Stevens, F. C. (1978) *Biochemistry 17*, 3924–3928.

Walsh, M., Stevens, F. C., Kuznicki, J., & Drabikowski, W. (1977) *J. Biol. Chem. 252*, 7440–7443.

Wang, C. L. A., Aguaron, R. R., Leavis, P. C., & Gergely, J. (1982) *Eur. J. Biochem. 124*, 7–12.

Watterson, D. M., Sharief, F., & Vanaman, T. C. (1980) *J. Biol. Chem. 255*, 962–975.