Volume 336, number 2, 368–374 FEBS 13399 December 1993

The structure of apo-calmodulin

A ^{1}H NMR examination of the carboxy-terminal domain

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Received 19 October 1993; revised version received 3 November 1993

The structure of the carboxy-terminal domain of bovine calmodulin, TR_2C, in the calcium-free form was investigated using two-dimensional ^{1}H NMR. Sequential resonance assignments were made using standard methods. Using information from medium and long range contacts revealed by nuclear Overhauser enhancement, the secondary structure and global fold were determined. The apo protein possesses essentially the same secondary structure as that in the calcium activated form of intact calmodulin. However, the secondary structural elements are rearranged so that the hydrophobic binding pocket is closed in the apo-form.

NMR; Calmodulin; Calcium; EF-hand; Activation

1. INTRODUCTION

The regulatory protein, calmodulin, is a member of a superfamily of Ca^{2+} binding proteins which includes troponin C and calbindin D_{9k}. The Ca^{2+} binding sites of these proteins share a common helix–loop–helix motif, also called an EF-hand [1] which usually occur in pairs. Calmodulin has four such Ca^{2+} binding sites which are arranged pairwise into two domains which in turn are separated by a predominantly helical central tether. Calmodulin acts in a calcium-dependent manner to activate other proteins necessary for a wide range of metabolic processes [2]. The binding of calcium to these EF-hands has been demonstrated to activate the protein and allow it to bind its target proteins.

A great deal of structural information has been obtained for the Ca^{2+} form of calmodulin from X-ray crystallography [3] and NMR spectroscopy [4], and for the Ca^{2+} form complexed with target peptides [5,6]. These studies have shown that both domains of the Ca^{2+}-loaded form of the protein have exposed hydrophobic patches which together comprise the binding site for target peptides. Based on comparison with the X-ray crystal structure of the homologous protein, troponin C, in the half-loaded state [7,8], it has been proposed that calcium activates calmodulin by inducing an opening of the binding sites to expose the hydrophobic patches that interact with target peptides [9]. Indeed, evidence exists to support the idea of the binding sites being closed in the apo form. For example, small hydrophobic molecules are found to bind much more tightly to the calcium form of calmodulin than to the apo form [10]. However, it has proven difficult to obtain high resolution structural information on apo-calmodulin from either X-ray crystallography or NMR spectroscopy.

In order to address the question of the structure of apo-calmodulin, we have undertaken a ^{1}H 2D-NMR study of one domain of calmodulin, designated TR_2C. The strategy of studying single domains enables one to lessen the problems inherent in studying large proteins by NMR, namely, spectral overlap and line broadening. The utility of this approach in gaining insights on the structure of large proteins has been well demonstrated [11]. This is especially true in cases where the domain comprises a semi-autonomous module in which there is minimal contact with other domains in the protein. This is the case for calmodulin, which resembles a dumbell in which the two globular domains are separated by a long, predominantly helical tether [3,4]. Also, it has been previously shown that these domains fully retain their Ca^{2+} binding activity and ability to bind small hydrophobic molecules [12]. Thus the biophysical properties of TR_2C are likely to be identical to its properties in intact calmodulin. By examining a single domain of the protein, spectral overlap is minimized and we are

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Abbreviations: TR_2C, carboxy-terminal domain of calmodulin; NMR, nuclear magnetic resonance; Phe, phenylalanine; Tyr, tyrosine; NOE, nuclear Overhauser enhancement; 2D, two-dimensional; COSY, correlated spectroscopy; R-COSY, Relay-COSY; 2QF, double quantum filtered; 2Q, double quantum spectroscopy; TOCSY, total correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy.

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able to obtain high resolution data from which details on the structure of apo-calmodulin can be obtained.

2. MATERIALS AND METHODS

2.1. *Sample preparation*

Recombinant apo-TR_2C from bovine calmodulin was expressed in *E. coli* and purified as described previously [13,14]. The purified TR_2C was dissolved to a concentration of 4 mM in ^1H_2O (10% ^2H_2O), pH 6.0 with 0.02% NaN_3 added, or in 99% ^2H_2O, pH 6.0 with 0.02% NaN_3 added. All experiments were performed at 28°C.

2.2. *NMR experiments*

A General Electric Omega 500 spectrometer operating at 500.13 MHz for ^1H was used for all 2D experiments. Standard pulse sequences were used to obtain phase-sensitive COSY [15,16], R-COSY [17], 2QF-COSY [18] and 2Q experiments [19]. TOCSY experiments [20,21] were performed with mixing times of 80 and 120 ms with modifications described by Rance [22] using the DIPSI-2 mixing sequence [23]. NOESY experiments [24] were performed as described by Bax [25] using a mixing time of 200 ms. The carrier was set on the ^1H_2O resonance for all experiments. For experiments in 90% ^1H_2O, the ^1H_2O resonance was suppressed by irradiation using the decoupler during a presaturation period of 1.3 s. A total of 512 spectra were collected for each experiment. Each spectrum is made up of 2048 complex data points taken from 64 accumulated scans using a spectral width of 6410 Hz.

Data were processed using the GE Omega software on a Sun 3/260 workstation equipped with an array processor. All spectra were zero-filled to 1024 by 2048 complex points. Phase-shifted squared sinebell window functions were applied in both dimensions to data which are cosine modulated. Unshifted sinebell window functions were used for sine modulated data. Assignment of the spectra was carried out using our inhouse MAGNE software. Baseline flattening was performed after transformation using a dispersive fit as described by Adler and Wagner [26], also using the MAGNE software.

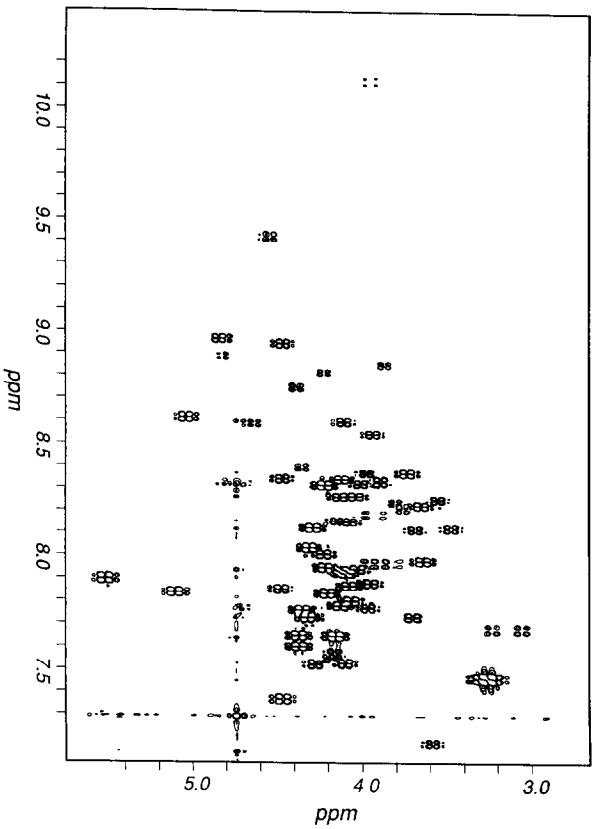


Fig. 1. A ^1H 2D COSY spectrum of apo-TR_2C in 90% H_2O, 10% ^2H_2O, pH 6.0, 28°C. The fingerprint region, consisting of backbone NH-C_αH contacts is shown.

3. RESULTS

Due to the size, internal homology and pseudo-symmetry of calmodulin, assignment of the Ca^{2+} form required the use of ^{15}N and ^{13}C labelled material and 3- and 4-dimensional experiments in order to overcome the severe overlap of resonances [4]. The considerable complexity of the ^1H NMR spectra of apo-calmodulin indicated that assignment would be even more difficult (data not shown). Thus, in order to overcome these difficulties, the resonance assignments were carried out on a single domain of calmodulin, TR_2C. The TR_2C domain is comprised of residues 76–148, which make up the third and fourth calcium binding sites of the protein.

^1H spin system assignments were carried out according to procedures described by Wüthrich [27] and Chazin et al. [28]. Using connectivities between backbone amide protons and sidechain protons observed in the COSY, R-COSY and TOCSY experiments it was possible to identify most of the spin systems. A representation of the NH-C_αH 'fingerprint' region of the COSY spectrum of apo-TR_2C is shown in Fig. 1. As can be seen, the resonances are well dispersed and all but a few of the backbone NH-C_αH cross peaks could be observed. This demonstrates the advantage of examining only one domain of apo-calmodulin and the possibility of obtaining high resolution structural information from the NMR data. The aromatic sidechains, 3 Phe and 2 Tyr, were identified from their characteristic shifts and connectivities and were related to their corresponding backbone resonances by connectivities in the NOESY spectra. Short and medium range d_{NN} and $d_{\alpha N}$ contacts derived from the NOESY spectrum were used to link spin systems sequentially. These connectivities are shown in Fig. 2.

Using medium and long range d_{NN} and $d_{\alpha N}$ connectivities observed in the NOESY spectrum, the secondary structure of apo-TR_2C can be defined. Based on the observation of series of series of $d_{\alpha N}(i,i+2)$ and $d_{\alpha N}(i,i+3)$ connectivities, four α-helices are identified at residues 79–96, 102–112, 117–129 and 137–147. These helices are arranged in pairs flanking the Ca^{2+} binding sites just as is observed in the Ca^{2+} form of calmodulin. In addition to the four α-helices, the small β-sheet formed by the two strands of the Ca^{2+} binding sites is also conserved in the apo protein. As shown in Fig. 3, long range d_{NN}, $d_{\alpha N}$ and $d_{\alpha\alpha}$ connectivities between residues 98–100 and 136–138, indicate the presence of the β-sheet in the same position as in the calcium loaded protein. The presence of β-structure had been suggested previously based on the observation of downfield

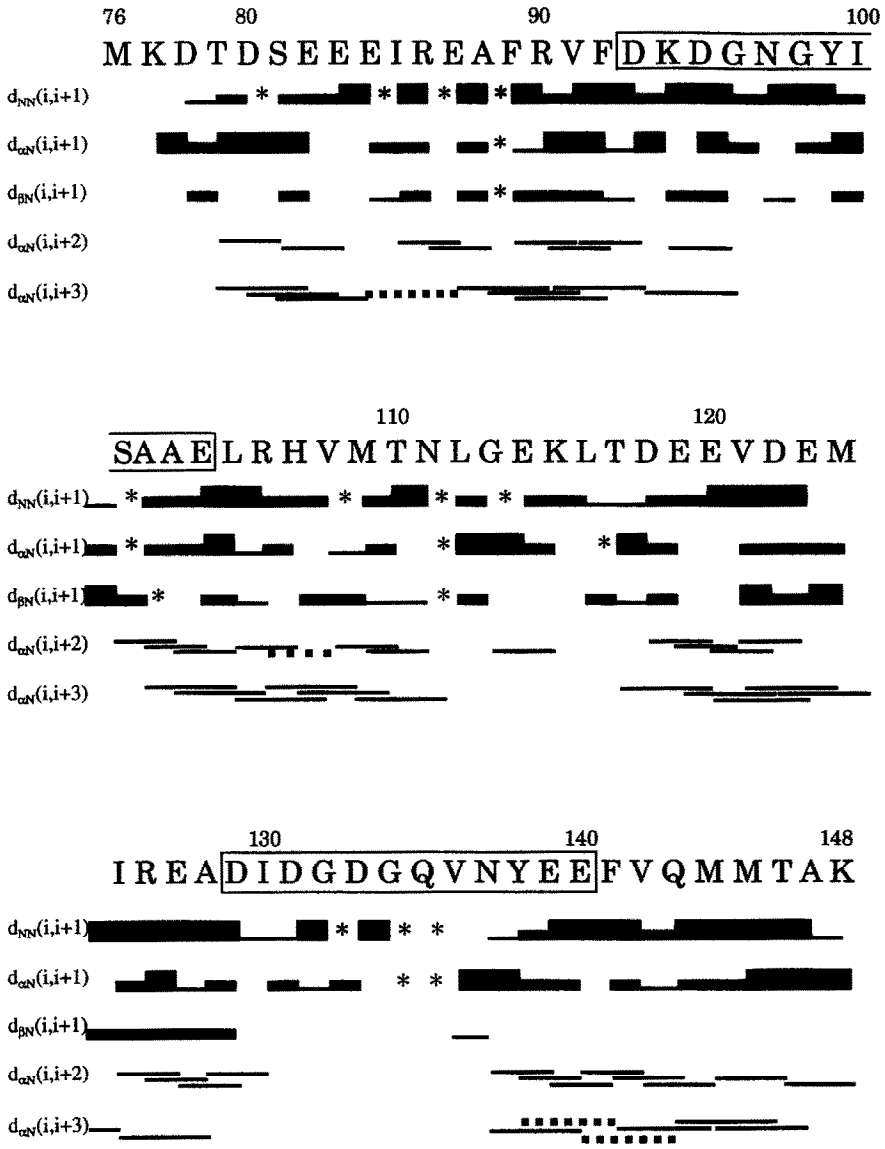


Fig. 2. The sequence of the TR_2C domain of bovine calmodulin with sequential (i, $i+1$) and medium range (i, ($i+2$ or $i+3$) connectivities indicated below. Relative bar height indicates the intensity of the crosspeak in the NOESY spectrum corresponding to the connection between the protons indicated. Dashed lines and * indicate connectivities that were unresolvable due to spectral overlap. The calcium-binding sites are enclosed in boxes.

shifted $C_\alpha H$'s in 1D spectra of apo-calmodulin domains [29]. However, the connectivities presented here provide the first direct evidence of the β-sheet and its location. Overall, the secondary structural elements of TR_2C are conserved in the apo-form. This is in good agreement with previous results obtained from circular dichroism spectroscopy [30].

Given the distribution and organization of the secondary structural elements in apo-TR_2C indicated by NOE contacts, it is readily apparent that the global fold of apo-TR_2C must, to a first approximation, resemble that of the calcium-loaded form of this domain in calmodulin. The two-stranded antiparallel β-sheet involving the strand between helices A and B and that between helices C and D, constrains the helices in a four-helix bundle arrangement. In the calcium loaded form, this bundle is divided into pairs of helices with helix A paired with helix D and helix B paired with helix C [3]. The helix pairs are separated from one another, leaving the hydrophobic residues on their interior faces exposed to solvent and available for binding to target molecules.

The apo-form, however, has been shown to be inactive, presumably due to sequestering of the active site hydrophobic side chains from the solvent. Using as a starting point the X-ray crystal structure of troponin C, in which one of two domains is calcium loaded while the other is not, model structures of apo-Troponin C and apo-calmodulin have been proposed [8,9]. These models predict a closure of the binding sites in the apo-forms in which the helix pairs are brought together such that

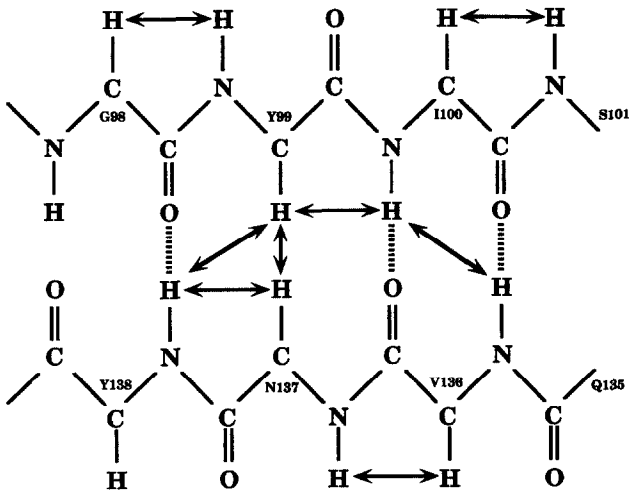


Fig. 3. A schematic diagram of the small two-stranded β-sheet. Arrows indicate those protons showing connectivities characteristic of β-sheet structure. The hatched lines indicate potential hydrogen bonds.

the hydrophobic residues are now in contact with each other rather than with the solvent. In order to directly test the model for apo-calmodulin, we can compare the predicted contacts formed by hydrophobic residues in the model apo-calmodulin, and their relative positions in the calcium loaded protein. In this way it is possible to identify pairs of hydrophobic residues which should exhibit NOEs in the apo-form but not in the calcium form.

Analysis of the NOESY spectrum of apo-TR_2C shows that many of the contacts between hydrophobic residues expected based on the model of apo-calmodulin are observed. Several of these are shown in Fig. 4. Many of the best resolved contacts are those between aromatic sidechains, especially Phe-89 in helix A and Phe-141 in helix D, and methyl protons of aliphatic sidechains Leu-105 and Val-108 which are well resolved in the region of 0.0 to 1.5 ppm. In Table I, these contacts, as well as others, are listed together with their interatomic distances in the model apo-calmodulin and Ca^{2+}-calmodulin structure. Keeping in mind that interproton NOEs are generally observed between protons separated by less than ~ 5.0 Å [27], the contacts listed in the table agree very well with the proposed model structure of apo-calmodulin with only minor exceptions.

Table I

Long-range interhelical NOEs observed in the apo-TR_2C domain of bovine calmodulin compared with distance in the model structure of apo-calmodulin and in the X-ray structure of Ca^{2+}-calmodulin

Residue 1	Residue 2	Helices	Distance apo (Å)[a,b]	Distance Ca^{2+} (Å)[a,c]
A88C_βH	V108$C_{\gamma 1}$H	A–B	3.8	8.0
F89NH	V108$C_{\gamma 1}$H		4.2	9.2
F89NH	V108C_βH		6.6	10.7
F89C_αH	V108$C_{\gamma 1}$H		3.2	7.5
F89C_αH	V108$C_{\gamma 2}$H		4.9	9.2
F89C_δH	L105$C_{\delta 1}$H		6.1	9.1
F89C_δH	V108$C_{\gamma 1}$H		3.0	10.1
F89C_δH	V108$C_{\gamma 2}$H		4.5	11.3
L105$C_{\delta 1}$H	F141C_δH	B–D	3.2	6.7
L105$C_{\delta 1}$H	F141CeH		3.6	6.7
L105$C_{\delta 1}$H	M144$_\beta$H		4.2	9.5
L105$C_{\delta 1}$H	M144$_\gamma$H		4.9	8.0
V108$C_{\gamma 1}$H	F141C_δH		5.2	9.0
V108$C_{\gamma 1}$H	F141C_εH		3.3	10.3
V108$C_{\gamma 2}$H	F141C_δH		6.0	10.4
V108$C_{\gamma 2}$H	F141C_εH		5.2	11.0

[a]For degenerate protons, the closest pair is reported.
[b]From coordinates of model apo-calmodulin [9].
[c]From X-ray crystal structure coordinates of Ca^{2+}-calmodulin [3].

4. DISCUSSION

Taking the information obtained from ^1H NMR together with that previously known about apo-calmodulin, we can make several conclusions about the structural transition induced in calmodulin by calcium acti-

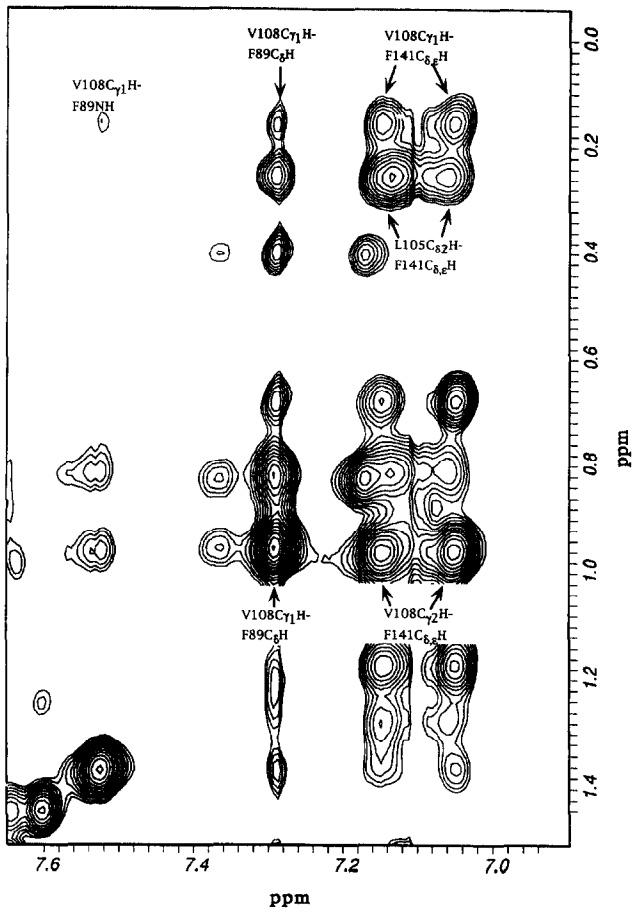
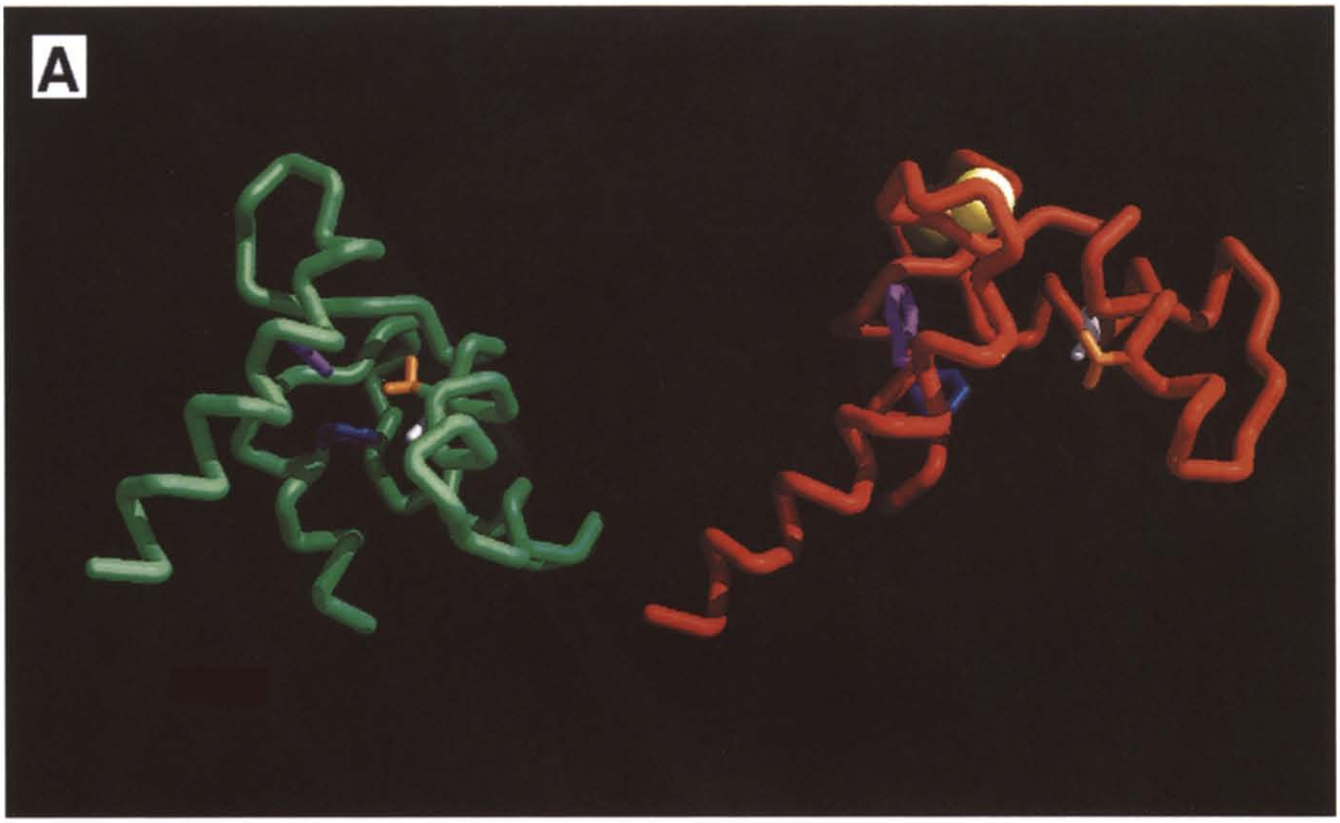
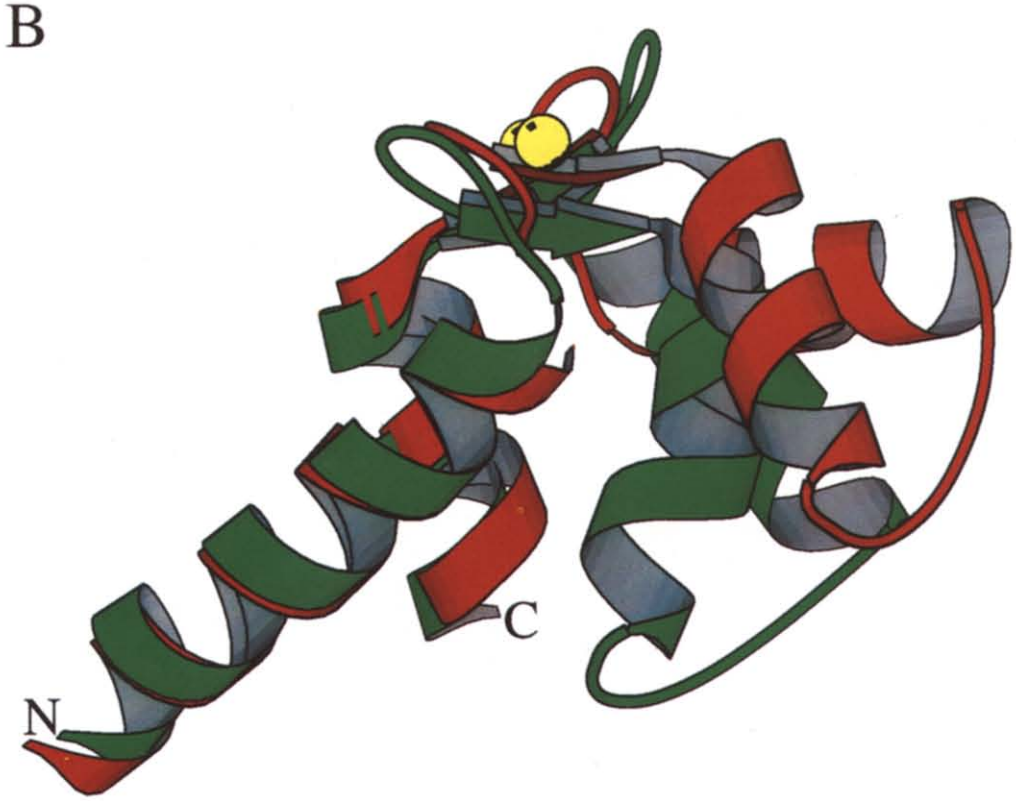


Fig. 4. A contour diagram of the NOESY spectrum of apo-TR_2C in 90% H_2O, 10% ^2H_2O, pH 6.0, 28°C with a mixing time of 200 ms. The region where several long-range aromatic-methyl connectivities characteristic of the apo form are observed is shown.


A


B
C
N

←
Fig. 5. (A) A tube drawing of the backbone of the TR_2C domain of the modelled apo-calmodulin [9] and of the X-ray structure of Ca^{2+}-calmodulin [3]. The apo form is colored green and the calcium form red. Several of the sidechains showing critical contacts in the apo form are also shown (Phe-89, purple; Leu-105, orange; Val-108 white; and Phe-141, blue). Figure made using MidasPlus [31]. (B) A ribbon diagram of the apo and calcium forms of TR_2C as in (A) but overlaid to show the large conformational change between the apo and calcium forms. The apo form is shown in green and the calcium form in red. Figure made using Molscript [32].

vation. Based on the data presented here and the medium and long range constraints determined so far, we can conclude that in the apo-form, TR_2C possesses essentially the same secondary structure as that in calcium loaded calmodulin. However, these elements of secondary structure are rearranged upon calcium binding so that the hydrophobic residues buried in the apo form are exposed to solvent and are available for binding target molecules. The model proposed previously for apo-calmodulin agrees very well with our results on the secondary structure and global fold. As shown in Fig. 5A, the hydrophobic sidechains which give rise to NOE connectivities in the apo form are quite close together in the model but are distant from each other in the Ca^{2+}-activated form of calmodulin.

In Figure 5B, the large conformational change proposed to occur upon calcium activation is shown. A similar conformational change in troponin C, first proposed by Herzberg and co-workers [8] has also recently been supported by NMR evidence [33]. However, this type of conformational change is by no means observed for all members this superfamily of calcium binding proteins. Calbindin D_{9k}, a smaller member of this superfamily of calcium binding proteins which possesses one pair of calcium binding sites, has been thoroughly investigated by X-ray crystallographic [34] and NMR methods [35,36]. High resolution structures of the calcium loaded and apo forms determined by NMR show that calbindin D_{9k} undergoes a relatively subtle conformational change upon calcium binding ([35]; N. Skelton, personal communication). That calcium-binding causes such a differential effect on two closely related proteins such as calbindin D_{9k} and calmodulin poses an interesting quandary in structural biology. The results presented above offer details about the conformational change induced by calcium binding in calmodulin. Further studies are underway to more precisely define the structure of apo-TR_2C, as well as the calcium loaded form, in order to gain more insight into the mechanism by which calcium activates calmodulin and other calcium-binding proteins.

Acknowledgements: We thank Eva Thulin for purification of recombinant apo-TR_2C and Drs. Tomas Grundström and Peter Brodin for supplying the recombinant TR_2C clone. We thank Mats Wikström for technical assistance and Peter Drakenberg for the MAGNE software. Thanks to Drs. Natalie Strynadka and Michael James for making the coordinates of the apo-calmodulin model available to us. This work was supported by the Swedish Natural Science Research Council (T.D., S.F., B.E.F.), the U.S. National Institutes of Health (B.E.F.), and the NESTE foundation (visiting scientist scholarship to T.D.). The Omega GN-500 spectrometer was purchased with grants from the Knut and Alice Wallenberg Foundation and the Swedish Council for Planning and Coordination of Research.

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