NMR studies of the methionine methyl groups in calmodulin

Kirsi Siivari[a], Mingjie Zhang[a], Arthur G. Palmer III[b], Hans J. Vogel[a]

[a]Department of Biological Sciences, University of Calgary, 2500 University Drive N.W., Calgary, Alberta, T2N 1N4, Canada
[b]Department of Biochemistry and Molecular Biophysics, Columbia University, 630 W. 168th St., New York, NY 10032, USA

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Abstract Calmodulin (CaM) is a ubiquitous Ca^{2+}-binding protein that can regulate a wide variety of cellular events. The protein contains 9 Met out of a total of 148 amino acid residues. The binding of Ca^{2+} to CaM induces conformational changes and exposes two Met-rich hydrophobic surfaces which provide the main protein-protein contact areas when CaM interacts with its target enzymes. Two-dimensional (^{1}H,^{13}C)-heteronuclear multiple quantum coherence (HMQC) NMR spectroscopy was used to study selectively ^{13}C-isotope labelled Met methyl groups in apo-CaM, Ca^{2+}-CaM and a complex of CaM with the CaM-binding domain of skeletal muscle Myosin Light Chain Kinase (MLCK). The resonance assignment of the Met methyl groups in these three functionally different states were obtained by site-directed mutagenesis (Met→Leu). Chemical shift changes indicate that the methyl groups of the Met residues are in different environments in apo-, calcium-, and MLCK-bound-CaM. The T_1 relaxation rates of the individual Met methyl carbons in the three forms of CaM indicate that those in Ca^{2+}-CaM have the highest mobility. Our results also suggest that the methyl groups of the unbranched Met sidechains in general are more flexible than those of aliphatic amino acid residues such as Leu and Ile.

Key words: Calmodulin; Methionine; Calcium; Mutant; Dynamics

1. Introduction

CaM is a ubiquitous, small, acidic Ca^{2+}-binding protein found in all eukaryotes. It plays a pivotal role by regulating numerous cellular events in a Ca^{2+}-dependent manner [1,2]. In the crystal structure the Ca^{2+}-saturated protein is a dumbbell-shaped molecule [3], however in solution the two domains of the protein are connected by a flexible, solvent-exposed linker region [4,5]. CaM contains 9 Met residues out of a total of 148 amino acids, this is much higher than the statistical average for the occurrence of Met residues in other proteins [6]. The binding of two Ca^{2+} ions to each domain of CaM induces significant conformational changes and exposes two Met-rich hydrophobic surfaces [2,3,7]. Of the 9 Met residues, four are located in each globular domain of CaM, while Met-76 is part of the central linker region of the protein [3]. The X-ray structure of Ca^{2+}-CaM reveals that all the Met residues in the two globular domains are located on the surface of the two hydrophobic patches, and they are all solvent accessible to varying degrees [3]. NMR and X-ray studies of complexes of CaM with various CaM-binding domain peptides show that all the Met residues, with the possible exception of Met-76, are in contact with the hydrophobic face(s) of the bound peptides [8–12]. Oxidation studies of CaM's Met residues have revealed that all of the Met residues can be readily oxidized in apo- and Ca^{2+}-CaM while they have a decreased accessibility upon the binding of the CaM-binding model peptide melittin [13]. These earlier studies indicate that the Met residues in CaM are in distinct microenvironments when the protein is in its three different physiological states, viz. apo-CaM, Ca^{2+}-CaM and complexes with its target enzymes. Recently, it was proposed that the Met residues of CaM are responsible for its ability to recognize a wide variety of target proteins in a sequence independent manner [6,14]. The high flexibility of the unbranched Met sidechain and the intrinsic polarizability of the sulfur atoms can provide a malleable, yet high affinity hydrophobic surface to accommodate various target enzymes. Indeed, we have found that the replacement of CaM's Met residues with Leu can reduce the protein's ability to activate its target enzymes such as cyclic nucleotide phosphodiesterase [12], calcineurin, and MLCK (unpublished results) to a different extent. Furthermore, by substituting the unnatural analog selenomethione for Met, evidence for the polarizability of the S and Se atoms in CaM has been obtained from ^{77}Se NMR experiments [15].

In this work, we have studied the Met methyl groups of CaM in its three different states, by selectively labelling the protein with [*methyl*-^{13}C]Met. The resonances of the Met methyl groups were studied in two-dimensional proton-detected heteronuclear NMR experiments and assigned to specific Met residues by site-directed mutagenesis (Met→Leu). It has been known for some time that natural abundance and isotope-labelled ^{13}C relaxation measurements can provide a unique insight into the motions of methyl groups in amino acid sidechains in proteins [16–19], (for more general reviews see [20,21]). In addition, carbon-13 relaxation lacks some of the disadvantages inherent in proton NMR relaxation studies [22]. The sensitivity of the ^{13}C relaxation studies can be improved by utilizing more sensitive proton-detected 2D NMR detection schemes [23–26]. Here we have determined the T_1 relaxation times for the labelled Met methyl carbons to gain information about the flexibility of the Met sidechains in CaM.

2. Materials and methods

CaM was overexpressed and purified from *E. coli* cells as described previously [27]. The Met→Leu mutants of CaM used in this work have been described in detail elsewhere [12]. The Met methyl-^{13}C-selectively labelled CaM and CaM mutants were prepared as reported earlier for selenomethionine-CaM [15]. The 22-residue synthetic MLCK peptide, which comprises the CaM-binding domain of the enzyme, was used as described before [28]. Three Met methyl-^{13}C-labelled CaM samples: apo-CaM (≈1.5 mM), Ca_4^{2+}-CaM (≈1.5 mM) and a complex of

Corresponding author. Fax: (1) (403) 289-9311.
Email: vogel@acs.ucalgary.ca

Abbreviations: CaM, calmodulin; MLCK, myosin light chain kinase; NMR, nuclear magnetic resonance; HMQC, heteronuclear multiple quantum coherence; nOe, nuclear Overhauser effect; 2D, two-dimensional; wt-CaM, wild type calmodulin.

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Ca_4^{2+}-CaM with the CaM-binding domain peptide of MLCK (≈1.0 mM), were prepared in D2O, pD 7.0, as described earlier [27].

2.1. NMR spectroscopy

All NMR spectra were acquired at 25°C on a Bruker AMX-500 spectrometer using a 5 mm inverse-detection broadband probe. The NMR data were processed on an X32 computer using the Bruker UXNMR software. Because the chemical shifts of the Met resonances are somewhat temperature dependent, we also obtained some spectra at other temperatures, to allow for comparisons. For 2D spectra, a 72°-phase-shifted sine-squared window function was applied in the F1 and F2 dimension before Fourier transformation. ($^1H,^{13}C$)-HMQC spectra were obtained using the pulse sequence of Bax et al. [29]. T_1 relaxation data for the Met methyl carbons were measured using the pulse sequence for 2D proton-detected ^{13}C relaxation described by Nicholson et al. [23]. This sequence is designed for methyl groups and cancels the cross correlation between the dipole–dipole and chemical shift anisotropy relaxation mechanisms. Six spectra with delays of 50, 150, 300, 600, 900, and 1500 ms were recorded and analyzed. The spectra were recorded with a total of 128 experiments with 32 scans per experiment. T_1 values were obtained by fitting the peak volumes, I, as a function of the relaxation delay, T, using the equation: $I = I_0 \exp(-T/T_1)$.

3. Results

3.1. Assignment of the Met methyl groups in apo-CaM, Ca^{2+}-CaM and the MLCK peptide-CaM complex

The assignment of the methyl groups from Met residues in homonuclear proton correlation NMR spectra of proteins is not always straightforward since the magnetization pathway to the methyl group is interrupted by the sulfur atom. Therefore, nOe-based NMR experiments are generally used to correlate the resonance of the methyl group of a Met to its own backbone [4], or alternatively heteronuclear NMR methods can be used [29]. Currently the assignments for the backbone and sidechain protons of apo-CaM and various target-peptide-bound forms of CaM are not available. Thus, we used a different strategy which would allow us in principle to assign the Met methyl resonances in any form of CaM. Our approach relies on a combination of site-directed mutagenesis and 2D ($^1H,^{13}C$)-HMQC NMR spectroscopy. Fig. 1A shows the HMQC spectrum of ^{13}C-selectively labelled Met methyl Ca_4^{2+}-CaM. It is obvious that the 9 Met methyl groups are well resolved in the spectra. The other panels in Fig. 1 provide examples of the assignment of individual Met methyl groups in Ca^{2+}-CaM using the M109L, M71L, and M144L mutants. The missing resonance in each panel is represented by an open box, and the assignments can be readily made from a comparison with the spectrum of wt-CaM. In this fashion we have assigned all the Met methyl groups in Ca^{2+}-CaM, and this assignment has been indicated in Fig. 1 (see also Table 1).

The same single mutant approach has also been used to assign the Met methyl groups in apo-CaM; Fig. 2 shows an example of the assignment of Met-144 in the spectrum of apo-CaM. It should be noted that the ($^1H,^{13}C$)-HMQC spectrum of

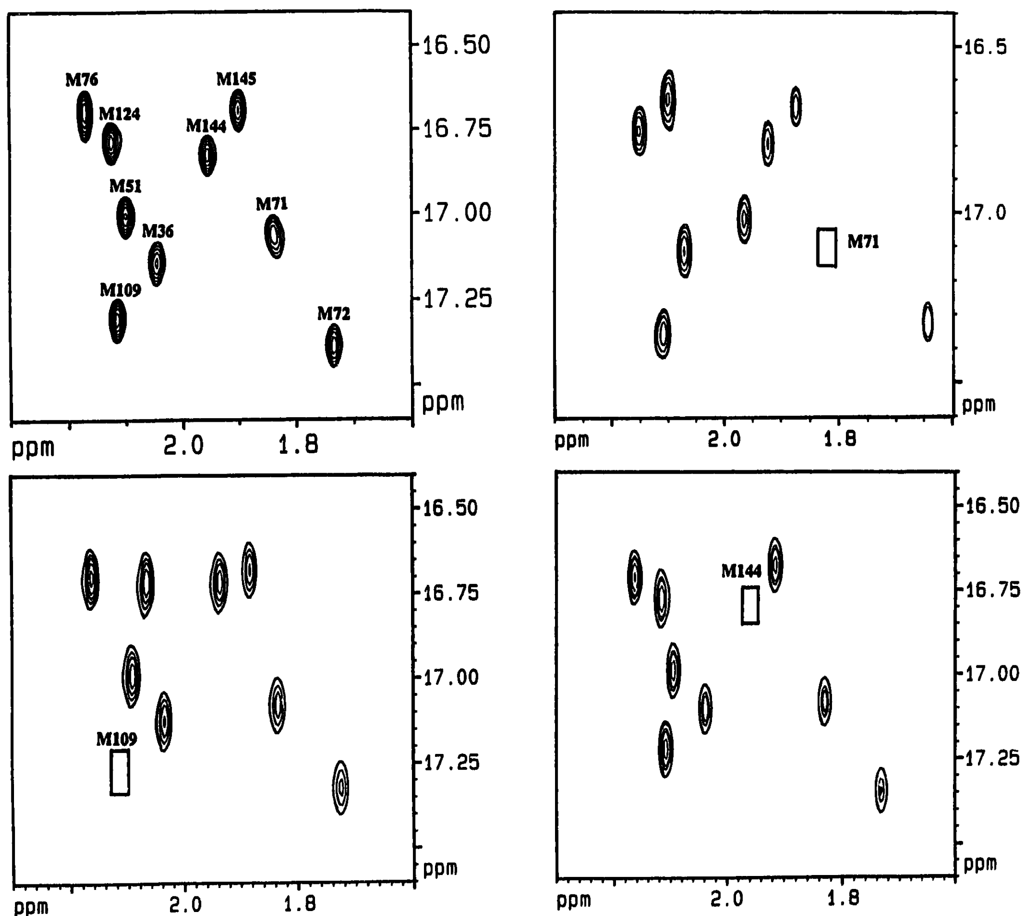

Fig. 1. ($^1H,^{13}C$)-HMQC NMR spectra of [*methyl*-^{13}C]Met-labelled wt-calcium-CaM (top left) and three Met→Leu mutants: M71L (top right), M109L (bottom left), and M144L (bottom right).

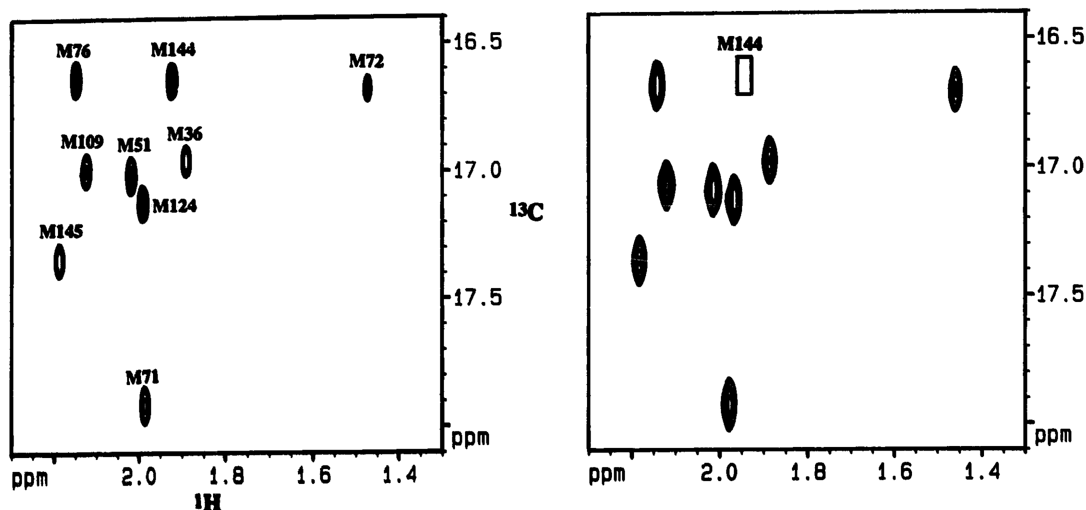

Fig. 2. (^{1}H,^{13}C)-HMQC NMR spectra of [*methyl*-^{13}C]Met-labelled apo-CaM, and its M144L mutant.

apo-CaM is markedly different from that of Ca^{2+}-CaM. None of the specific Met→Leu mutants gave rise to major spectral changes for any of the other Met resonances in the spectra of apo-CaM. Thus the complete assignment for apoCaM could be obtained without ambiguity. Likewise only small changes were observed for the four C-terminal Met residues of Ca^{2+}-CaM, upon introducing a Met→Leu mutation in this domain of the protein. However, mutation of a Met residue in the N-terminal domain of Ca^{2+}-CaM gave rise to significant perturbations of the resonances of the other three Met residues in the domain (see for example Fig. 1, top right panel). In order to ascertain that the correct assignment for the Met methyl groups in the N-terminal domain was obtained, an apo-CaM sample was titrated with Ca^{2+}. Since Ca^{2+} binds in fast exchange to the N-terminal domain of CaM, it was possible to follow the movement of the four N-terminal Met methyl resonances without ambiguity during the titration; this experiment has confirmed the assignment indicated in Fig. 1. The titration experiment also confirmed that the first two equivalents of CaM bind in slow exchange and with positive cooperativity to the C-terminal domain and only change its conformation. The third and fourth equivalent of Ca^{2+} bind to the N-terminal domain in fast exchange, and in the absence of a target peptide only change its conformation (for review see [2,7]).

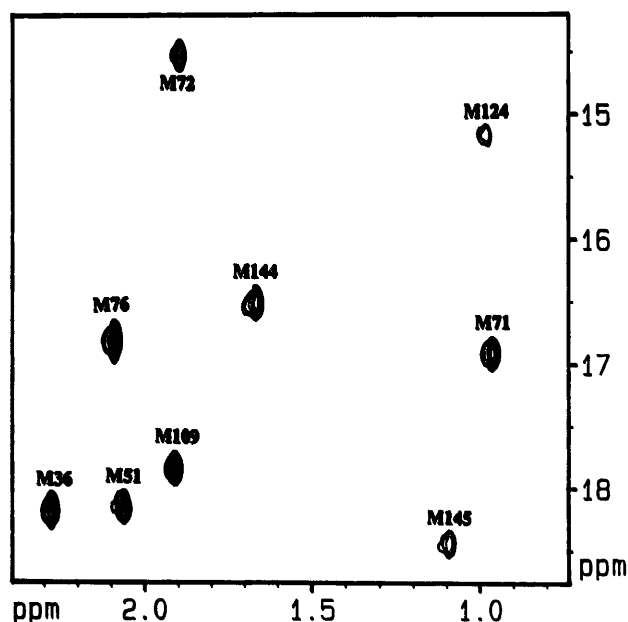

Fig. 3. (^{1}H,^{13}C)-HMQC NMR spectrum of selectively labelled CaM complexed with the MLCK peptide.

Fig. 3 shows the (^{1}H,^{13}C)-HMQC spectrum of the selectively labelled CaM complex with the CaM-binding domain of MLCK. As expected, nine resonances representing the 9 Met methyl groups from CaM are observed. The assignment of the Met methyl groups in the complex was also obtained with the aid of the single mutant proteins used for the assignments of the Ca^{2+}- and apo-forms of CaM (see Table 1). Compared to apo- and Ca_4^{2+}-CaM the Met methyl groups in the complex display a much wider chemical shift dispersion in both the ^{1}H and ^{13}C dimensions. With the exception of Met-76, all of the Met residues undergo chemical shift changes when CaM changes from the apo-form to the Ca^{2+}-form and subsequently to the MLCK-peptide bound form. This suggests that each Met residue is in a different microenvironment in the three physiologically distinct states of the protein.

Table 1
Chemical shifts (ppm) of the Met methyl groups in different forms of CaM

Residue	apo-CaM		Ca^{2+}-CaM		CaM/MLCK	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
M36	16.45	1.89	17.10	2.04	18.28	2.27
M51	17.02	2.02	16.98	2.10	18.20	2.04
M71	17.92	1.98	17.16	1.83	16.92	0.96
M72	16.67	1.48	17.34	1.73	14.51	1.89
M76	16.64	2.15	16.70	2.17	16.78	2.09
M109	17.00	2.12	17.33	2.12	17.80	1.91
M124	17.12	1.99	16.81	2.12	15.22	0.98
M144	16.64	1.92	16.81	1.96	16.51	1.66
M145	17.34	2.18	16.71	1.90	18.43	1.08

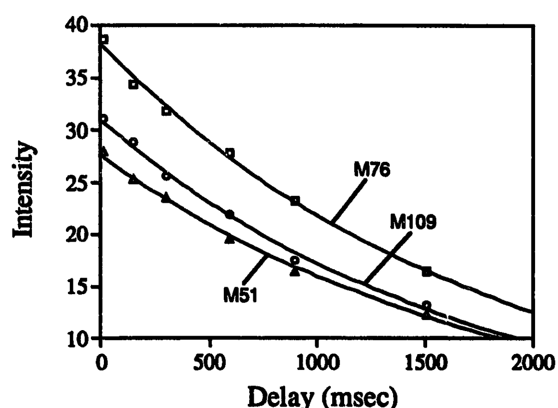

Fig. 4. T_1 decay curves obtained for Met-51, Met-76 and Met-109 in apo-CaM. The curves drawn represent a least-squares fit using a single exponential decay.

3.2. T_1 relaxation data

Fig. 4 shows representative T_1 relaxation decay curves obtained for several methyl groups. No significant deviations from a single exponential decay were observed for the T_1 relaxation data. Table 2 lists the T_1 relaxation rates of Met methyl carbons in apo-CaM, Ca^{2+}-CaM and the CaM-MLCK-peptide complex.

4. Discussion

The sidechains of the Met residues of CaM play an important role in the function of this ubiquitous regulatory calcium-binding protein [2,6,8,12,14]. It was therefore important to us to develop a sensitive and relatively simple NMR method for studying these residues. The approach chosen should not only be applicable to comparing the forms of CaM for which complete backbone assignments are available, but should allow the study of a range of complexes of CaM with its many target proteins and peptides. Therefore, we used a combination of specific isotope-labelling with relatively inexpensive [*methyl*-^{13}C]Met, two-dimensional proton-detected carbon-13 spectra for enhanced sensitivity and resolution, as well as site-directed mutagenesis of all Met residues of CaM. The results presented here show the success of this approach. All Met residues gave rise to well resolved resonances, that could be readily assigned by comparison of the NMR spectra of mutant proteins and the wild type protein. While the local perturbations introduced by the various Met→Leu substitutions appeared minimal in apo-CaM and the MLCK-CaM-complex, the N-terminal domain of Ca^{2+}-CaM was perturbed somewhat by the mutations, necessitating additional titration experiments to confirm the assignments. The availability of this group of Met mutants has further allowed us to study the role of the Met residues in other complexes of CaM, such as cyclic nucleotide phosphodiesterase [12], caldesmon [11], and nitric oxide synthase (unpublished results), illustrating its wide applicability. In a recent communication, Putkey and coworkers have shown that a similar approach, involving multiple Met mutations, can also be used successfully in the case of the homologous protein cardiac troponin C [30]. Recently, a detailed assignment for the Met methyl groups in calcium-CaM has been published [4]. Except for an interchange of Met-144 and Met-145, the assignments obtained in this work are identical [4]. The latter and our results are however at variance with earlier published assignments for the Met residues in calcium CaM [31]. Our assignments for the CaM-MLCK complex are consistent with those in [29]; the assignments for apo-CaM have not been reported yet.

The apo-form of CaM is not capable of activating target proteins; therefore the binding of Ca^{2+} to CaM is thought to expose the two Methionine-rich hydrophobic surface regions of CaM [6,7]. These two regions of CaM are essential for binding the target proteins [2]. Our data show that the chemical shifts of the Met methyl resonances are quite distinct in the three functional states of CaM (see Table 1). Clearly the Met residues in apo-CaM are in a different environment from those in calcium-CaM. Binding of a target peptide to the two hydrophobic regions causes a large distribution of chemical shifts, indicating that the Met residues are experiencing even more widely different environments in this state; this was also noted in the case of the complex of CaM with a phosphodiesterase peptide [12]. Further information about the dynamic behaviour of the Met residues can be obtained from their T_1 relaxation data (see Table 2). All the Met methyl motions are fast with respect to the overall rotational motion of the protein. The T_1 relaxation rates of the Met residues in calcium-CaM are in general longer than those in the two other states. This indicates that the Met residues are more flexible in this state, which is consistent with their relatively high solvent exposure in the calcium-form of the protein [3]. Interestingly, the T_1 values in apo-CaM are all shorter, indicating more hindered motions of the Met sidechains in this case. In the CaM/MLCK complex some Met are restricted, while others retain a high flexibility; nonetheless, it is known that all Met sidechains interact with the bound MLCK peptide [8]. In principle, it is possible to obtain more detailed information about the motions in the Met side chains, by analyzing T_1, T_2 and nOe relaxation data simultaneously using for example the model-free approach proposed by Lipari and Szabo [32,33]. In preliminary experiments, we have obtained the required additional data and performed such calculations [24,25] (data not shown). We have found that exchange processes, anisotropic motions, and other potential complications gave rise to ambiguous results for three Met residues in the CaM peptide complex. However the large majority of the Met methyl groups in the three forms of CaM had S^2 (order parameters) < 0.078 and τe (correlation times) < 10 ps. By comparison to the outcome of other recent studies addressing amino acid methyl motions in proteins [23,25], these values indicate a higher flexibility for the unbranched Met

Table 2
T_1-relaxation data (s) of the Met methyl carbons in the different forms of CaM*

Residue	apo-CaM	Ca^{2+}CaM	CaM/MLCK
M36	1.78	2.10	1.98
M51	1.60	2.14	2.02
M71	1.33	2.01	2.10
M72	1.58	1.98	2.78
M76	1.80	2.03	1.71
M109	1.66	2.02	1.47
M124	1.78	2.36	2.00
M144	1.64	2.30	1.59
M145	1.66	1.92	2.06

*At 25°C the overall rotational correlation time for apo-, and Ca^{2+}CaM is 8 ns, for the complex it is 10 ns.

sidechains in CaM, than for similar branched aliphatic side chains such as Leu and Ile. This is in agreement with the suggestion that Met sidechains have unparalleled flexibility [14], and that this feature contributes to CaM's capacity to interact with CaM-binding domains of widely different amino acid sequence, thus providing a partial rationale for the high Met content of the two hydrophobic interaction surfaces of CaM.

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