The Calmodulin-Binding Domain of Caldesmon Binds to Calmodulin in an α-Helical Conformation†

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ABSTRACT: The binding of calcium–calmodulin (CaM) to caldesmon (CaD) contributes to the regulation of smooth muscle contraction. It has been reported that a 17-residue synthetic peptide encompassing the residues Gly651–Ser667 of smooth muscle CaD constitutes its CaM-binding domain [Zhan, Q., Wong, S. S., & Wang, C. L. A. (1991) *J. Biol. Chem. 266*, 21810–21814]. This peptide does not share sequence homology with the CaM-binding domains of other proteins, and in addition, the binding of CaM to CaD is known to be relatively weak. Here we have investigated the properties of this atypical CaM-binding domain by NMR and circular dichroism (CD) spectroscopy. Two dimensional NMR studies performed in an aqueous TFE mixture (75%/25%) showed that the peptide has the capacity to adopt an amphiphilic α-helical conformation. TRNOESY experiments and CD spectroscopy were used to determine that the CaD peptide binds in an α-helical conformation to CaM. The addition of TFE or the binding of the CaD peptide to CaM induces an α-helical structure only for the central 10 amino acid residues of the peptide. Titrations of CaM with the CaD peptide were followed by proton NMR and show the formation of a 1:1 complex and that the binding is calcium-dependent. The chemical shifts of ^{13}C-methyl groups of specifically labeled Met residues and of the ^{15}N backbone amide groups of CaM undergo changes upon addition of the CaD peptide; these data suggest that both domains and the central helix of CaM are involved in the binding of the peptide. A possible mode of binding of the CaD peptide to the methionine-rich regions of CaM, which is consistent with these data, is discussed.

Caldesmon (CaD)[1] is a major component of the thin filaments in smooth muscle and nonmuscle tissues. It can bind to all of the major proteins that make up the actomyosin system, such as actin, myosin, and tropomyosin; in addition, it interacts with the ubiquitous calcium regulatory protein calmodulin (CaM). The actomyosin system uses ATP to generate mechanical force, hence caldesmon can play a role in contractile and motile events in smooth muscle and nonmuscle cells. CaD inhibits the actomyosin ATPase *in vitro* and *in vivo*; this inhibition can be attenuated by Ca^{2+}–CaM. It has also been shown that caldesmon can be phosphorylated by a variety of protein kinases; these transient modifications modulate its interactions with the other actomyosin proteins. Thus, through the direct action of CaM and through changes in the phosphorylation state, CaD plays a regulatory role in the contractile events in smooth muscle and nonmuscle tissue [for reviews, see Sobue and Sellers (1991), Marston and Redwood (1991), and Walsh (1991)].

Smooth muscle caldesmon is an elongated molecule which has an unusual domain structure (Wang et al., 1991b; Marston & Redwood, 1991). The role of the various domains has been investigated by domain mapping studies using proteolytic fragments and expressed truncated proteins. The binding sites for myosin are located in the N-terminal domain, while sites for actin, tropomyosin, and calmodulin are found in the C-terminal domain (Sobue & Sellers, 1991; Marston & Redwood, 1991). Of particular interest for this study are the findings of Bartegi et al. (1990) and Wang et al. (1991a); their results show that the region between Trp659 and Phe665 forms part of the CaM-binding site of CaD. This suggestion is also consistent with fluorescence studies, which implied that one or more Trp residues were involved in the CaM binding site (Shirinsky et al., 1988). These findings prompted Zhan et al. (1991b) to synthesize a 17-residue peptide which encompassed the region from Gly651 to Ser667. They subsequently showed that this peptide binds in a calcium-dependent manner to CaM with a K_D comparable to the native protein. The same peptide can also bind to actin, from which it is dissociated upon addition of Ca^{2+}–CaM; this resembles the way in which the native protein acts (Sobue et al., 1981). Like most other CaM-binding domains, the CaD peptide comprises mainly basic and hydrophobic amino acids. However, it does not have any obvious sequence homology with other CaM-binding domains, and it appears to be shorter. In addition, it also has a lower affinity for CaM with a K_D in the micromolar range, while the CaM-binding domains of most other proteins bind with nanomolar affinity. Because of this relatively weak binding, high concentrations of CaM are necessary to saturate the attenuating effect on actomyosin activity (Szpacenko et al., 1985). Hence, in view of the levels of CaM that are present in the cell (Kamm & Stull, 1989), the question has been raised whether this interaction plays a role under physiological conditions. However, recent *in vivo* cross-linking and force development studies, as well as *in vitro* kinetic studies provide support for the involvement of the CaM/

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[1] Abbreviations: CaD, caldesmon; CaM, Ca^{2+}–calmodulin; CD, circular dichroism spectroscopy; HMQC, heteronuclear multiple quantum coherence; HSQC, heteronuclear single quantum coherence; HPLC, high-pressure liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; MLCK, myosin light chain kinase; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy; TFE, trifluoroethanol; TOCSY, total correlated spectroscopy; TRNOE, transferred nuclear Overhauser enhancement; 1D, one dimensional; 2D, two dimensional.

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CaD interaction in the control of smooth muscle contraction (Mangels & Gregy, 1992; Katsuyama et al., 1992; Kasturi et al., 1993).

Because of the distinct nature of the CaM-binding domain of CaD, it was of interest to us to obtain information about its CaM-bound conformation. Primarily on the basis of CD studies, secondary structure predictions, and chemical modification studies, it has been established that most peptides bind to CaM in an α-helical conformation [see, for example, Malencik and Andersson (1984), Cox et al. (1985), Vogel (1987), and O'Neil and DeGrado (1990)]. Thus, as a first step toward determining whether the CaM-binding domain of CaD could adopt an α-helical conformation, we have studied its structure in the helix-promoting solvent trifluoroethanol (TFE) by NMR (Zhang et al., 1993). Recently, the three-dimensional structures of complexes of CaM with two MLCK CaM-binding domains were determined by NMR and X-ray methods (Ikura et al., 1992; Meador et al., 1992). These studies and related work have demonstrated that it is possible to determine the structure of a bound CaM-binding domain peptide by NMR. However, such NMR studies require the use of isotopically labeled protein or peptides (Ikura & Bax, 1992; Ikura et al., 1992; Roth et al., 1991), and they are generally performed under conditions of slow exchange on the NMR time scale (i.e., tight binding). While this latter condition is fulfilled for CaM-binding domains that bind with nanomolar affinities such as MLCK, the weaker bound CaD peptide is clearly not in slow exchange (see below). Fortunately, another NMR method, commonly referred to as transferred NOE, can be utilized under these conditions. This method relies on the exchange between the peptides in the free and bound state, which can transfer NOE information about the protein bound state to the free peptide resonances, where they can be more easily measured because of the larger amount of the free peptide and the narrower line widths of the peptide resonances. This approach has been successfully used to study the bound conformation of various linear peptides [see, for example, Meyer et al. (1988), Milen et al. (1990), Ni et al. (1990), Campbell and Sykes (1991), and Landry and Gierasch (1991)]. Here we report the outcome of TRNOE studies, which show that the CaD peptide binds to CaM in an α-helical conformation. In an attempt to obtain information about the regions of CaM that are involved in the binding of the peptide, we have also studied complexes of the CaD peptide with unlabeled CaM, with ^{13}C-methyl-Met-labeled CaM, and with uniformly ^{15}N-labeled CaM. Chemical shift changes were followed in ^{1}H, ^{13}C, and ^{15}N NMR spectra, and these suggest that the CaD peptide binds to similar regions of CaM as the MLCK CaM-binding domain.

MATERIALS AND METHODS

The 17-residue peptide (GVRNIKSMWEKGNVFSS) was synthesized by the Core Facility for Protein/DNA Chemistry at Queen's University, Kingston, Canada. The purity of the peptide was greater than 95% as judged by HPLC and amino acid analysis. The concentration of the peptide was determined by UV absorption using the extinction coefficient for the single Trp residue ($\epsilon_{280}^{Trp} = 5.6 \times 10^3$ cm^2 mol^{-1}) in the peptide. D_2O and deuterated TFE-*d* (CF_3CD_2OD) were obtained from MSD Isotopes, Montreal, Canada.

Expression and Purification of CaM. An *Escherichia coli* expression system was employed to overproduce CaM. In this system, a "run-away" plasmid containing a synthetic bovine CaM gene was transformed into *E. coli* MM294 cells (Waltersson et al., 1993). The cells harboring the plasmid were grown at 30 °C in LB media until the OD_{600} reached $\approx$1.5; subsequently the temperature of the culture was raised to 37 °C. At this point, IPTG up to a final concentration of 5 mM was added to the culture to induce the expression of CaM. Typically, 3–4 h of incubation at 37 °C would produce the maximum amount of CaM (Zhang & Vogel, 1993). Bacterially expressed CaM was purified using Ca^{2+}-dependent phenyl-Sepharose chromatography (Gopalakrishna & Anderson, 1982; Vogel et al., 1983; Putkey et al., 1985). The purified CaM appears as a single band on SDS–polyacrylamide gel electrophoresis. The concentration of CaM was determined by using the absorption coefficient of $E_{276}^{1\%} = 1.8$.

Carbon-13 Selective Labeling of CaM. Selective labeling of the methionine methyl groups in CaM with carbon-13 was carried out in chemically defined MOPS medium (Neidhardt et al., 1974). An 800-mL cell culture was initially grown at 30 °C in MOPS minimal medium supplemented with all the individual amino acids, except Met, at a concentration of 100 mg/L. When the OD_{600} of the culture reached $\sim$1.5, 200 mL of prewarmed ($\sim$65 °C) medium was added to the culture to bring the temperature to 37 °C. At the same time, 50 mg of $^{13}CH_3$-S-Met, 160 mg of IPTG, and 200 μL of a 50 mg/mL ampicillin stock solution were added into the culture. The cells were maintained at 37 °C in the shaker for another 3–4 h before harvesting. The $^{13}CH_3$-Met-labeled CaM was then purified following the same procedures described for unlabeled CaM.

Nitrogen-15 Uniform Labeling of CaM. Nitrogen-15 uniformly labeled CaM was obtained by expressing CaM in M9 minimal medium using $^{15}NH_4Cl$ (1 g/L) as the sole nitrogen source. A total of 50 mL of M9 medium containing $^{15}NH_4Cl$ was inoculated with 1 mL of an overnight cell culture grown in LB medium at 30 °C and was grown at 30 °C overnight. Subsequently, it was introduced as an inoculum into 500 mL of M9 medium, after which the cells were grown to OD_{600} $\sim$1.0. At this point the temperature of the culture was raised to 37 °C by heating the culture flask with hot tap water, 80 mg of IPTG was added into the culture, and it was incubated at 37 °C with aeration for 5 more h before harvesting. A concentration of 50 μg/mL of ampicillin was used throughout the various culture stages. The ^{15}N uniformly labeled protein was purified in the same way as unlabeled CaM.

Sample Preparations for NMR Studies. For the NMR studies of the peptide in TFE/water mixture, two samples (one in 75% H_2O/25% TFE-*d* and the other one in 75% D_2O/25% TFE-*d* were prepared. The concentration of the peptide in both samples was about 5 mM; the pH values of the samples were adjusted to 6.5 or 5.0 by the addition of the appropriate amount of 0.05 M KOD or DCl. pH measurements were not corrected for the solvent isotope effect.

For the TRNOE experiments, the peptide was first dissolved in about 0.4 mL of H_2O (or D_2O); then the pH of the peptide solutions was adjusted to 6.5 by adding dilute KOD or DCl. Subsequently, the required amount of a Ca^{2+}–CaM stock solution (1.5 mM, pH or p*D* 6.5) was mixed with the peptide solutions. The final concentration of the peptide and CaM in both samples was about 4.5 and 0.15 mM, respectively, which corresponds to a CaM:peptide ratio of 1:30. The final volume of both samples was about 0.45 mL.

For the (^{1}H,^{15}N)-HSQC experiment, 450 μL of a 1.4 mM ^{15}N uniformly labeled Ca^{2+}–CaM sample was prepared in a 90% H_2O/10% D_2O mixture in the presence of 0.1 M KCl. The pH of the sample was adjusted to 6.5. The complex of ^{15}N uniformly labeled CaM with the CaD peptide at a ratio

of 1:1.1 was prepared following the method described by Seeholzer and Wand (1989) with minor modifications. About 10 mg of ^{15}N uniformly labeled CaM was dissolved into H_2O, giving a final concentration of 0.2 mM. Then 0.9 mM of Ca^{2+} was added to saturate CaM with Ca^{2+}. The pH of the CaM solution was adjusted to 6.5 using 0.1 M HCl or KOH. Following pH adjustment, 11 μL of a 4 M KCl stock solution was added to the above CaM solution. Finally, the appropriate amount of the CaD peptide stock solution (1 mM, pH 6.5) was added dropwise into the CaM solution with gentle mixing. The final ratio of CaM to the peptide was 1:1.1. The diluted solution was concentrated on a spin vacuum drying system without freezing to a final volume of 400 μL. A total of 40 μL of D_2O was added to the concentrated solution to provide a spectrometer lock, and the pH of the sample was checked to be 6.5. The final concentration of Ca^{2+}–CaM was ~1.4 mM, and the final salt concentration was 0.1 M KCl.

NMR Spectroscopy. All the NMR spectra were acquired on a Bruker AMX500 spectrometer equipped with a 5-mm inverse detection probe. For experiments with the peptide in the TFE/water mixture, a temperature of 288 K was chosen to avoid resonance overlap of some of the α-protons with the water signal. For the TRNOE experiments, the spectra were recorded at 288 and 280 K. The pure-phase absorption mode of all the spectra was obtained by using the time proportional-phase increment (TPPI) technique (Marion & Wuthrich, 1983). All spectra were acquired with a deuterium lock from D_2O or TFE-*d* in the samples.

All of the 1D ^1H spectra were recorded with a sweep width of 6000 Hz, 16K data points, and 128 scans per experiment. In the NOESY spectra (Bodenhausen et al., 1984), a total of 512 FIDs were collected with a 5500-Hz sweep width and 2048 points in the F2 dimension. The mixing time used in the NOESY experiments was 200 ms for the peptide in TFE/water mixture and 300 ms for the peptide in pure water solution. TRNOESY spectra of the CaD peptide/CaM mixture were recorded with 100 and 200 ms mixing times to establish that cross-peaks did not arise from spin diffusion. TOCSY spectra were recorded using a standard pulse scheme (Bax & Davis, 1985) with a mixing time of 70 ms. The (^1H,^{13}C)-HMQC spectrum of the CaD peptide in 75% D_2O/25% TFE-*d* was acquired according to the method of Bax et al. (1983). For the spectra recorded in H_2O, a weak selective presaturation pulse covering a width of ~25 Hz was applied to suppress the H_2O signal. All the spectra were processed on an X32 computer using the Bruker UXNMR software package. For the 2D spectra, one time-zero filling was applied in F1, and a sine-squared window function with a 60° phase shift was used before Fourier transformation.

(^1H,^{15}N)-HSQC spectra (Bodenhausen & Ruben, 1980) were acquired at 30 °C for the ^{15}N uniformly labeled CaM and CaM/peptide complex. In each spectrum, 400 experiments with 32 scans for each experiment were recorded covering 25 ppm in the ^{15}N dimension and 12 ppm in the ^1H dimension.

CD spectroscopy was performed on a Jasco J-500 spectropolarimeter using a cell path length of 1 mm. The concentration of the peptide in the CD measurements was 13 μM; the buffer used was 5 mM citric acid (pH 5.0). The CD spectra of CaM and the CaM/peptide complex were obtained at 11 μM of CaM in 5 mM Tris buffer (pH 7.5), with either 0.5 mM Ca^{2+} or 2 mM EDTA added.

RESULTS

The sequence of the 17-residue CaD peptide, GVRNIKSMWEKGNVFSS, corresponds to the amino acid residues from Gly651 to Ser667 in caldesmon. The CaD peptide is believed to contain the CaM-binding domain of CaD, as it shows Ca^{2+}-dependent binding to CaM. It binds with 1:1 stoichiometry, and the K_D of the peptide/CaM complex has been determined by fluorescence spectroscopy using a fluorescent probe attached to the peptide (Zhan et al., 1991); the value obtained was ≈1 μM.

Two-dimensional ^1H NMR studies of the free CaD peptide in pure water were recorded at pH 6.5 and 5.0 (288 K). The results showed that the peptide does not have any regular secondary structure as judged from the lack of characteristic NOEs for regular helical or turn structure of linear peptides. Only $d_{\alpha N}(i,i+1)$ cross-peaks were observed, indicative of extended structures (Dyson & Wright, 1991). The CD spectrum of the CaD peptide in water also showed that the peptide has no detectable α-helical or turn-type structure (data not shown).

The CaD Peptide Adopts an α-Helical Structure in 25% Aqueous TFE. Since the CaD peptide does not have any regular secondary structures in pure H_2O, the capacity to induce secondary structure by the helix-promoting solvent TFE was studied by CD spectroscopy. The addition of TFE induced small but significant changes in the CD spectra of the peptide.[2] Once the concentration of TFE exceeded 25% (v/v), the CD spectrum of the peptide remained unchanged. Thus, in the subsequent NMR studies, a 25% TFE-*d*/75% H_2O (or D_2O) solvent mixture was chosen to investigate the conformation of the CaD peptide. The sequential assignment of the peptide was obtained by using the standard 2D ^1H NMR techniques for peptides and proteins (Wüthrich, 1986). The NH/NH region of the NOESY spectrum of the peptide in the 75% H_2O/25% TFE-*d* mixture revealed a substantial amount of strong $d_{NN}(i,i+1)$ cross-peaks (data not shown). This may indicate that a "nascent" helix can form in this peptide (Dyson & Wright, 1991). Further evidence for helix formation can be provided by the observation of medium range NOEs. Indeed some weak $d_{\alpha\beta}(i,i+3)$ cross-peaks could be observed in a NOESY spectrum recorded in 75% D_2O/25% TFE-*d* (data not shown). Figure 1A provides a summary of the pattern of the NOEs observed for the peptide. The presence of $d_{NN}(i,i+1)$ and $d_{\alpha\beta}(i,i+3)$ NOE cross-peaks suggests that the peptide adopts an α-helical structure from residues I5 to V14. The amide exchange rate at pH 5.0 is lower than at pH 6.5, thus the spectra recorded at pH 5.0 were slightly better. However, changing the pH from 5.0 to 6.5 will not change the net charge of the CaD peptide; hence, the structure obtained for the peptide at pH 6.5 and 5.0 should be identical. Indeed, the NMR spectra of the peptide recorded in the same solvent at pH 6.5 provided the same structural information as that obtained at pH 5.0 (data not shown).

The presence of an α-helical structure in a peptide can be further supported by the observation of secondary chemical shifts of the α-protons and α-carbons in the peptide. These are obtained by calculating the difference between the actual and the random coil chemical shifts. The ^{13}C chemical shifts of the α-carbons in the peptide were obtained by recording a natural abundance (^1H,^{13}C)-HMQC spectrum of the peptide in the 75% D_2O/25% TFE-*d* mixture (Figure 2). The secondary chemical shifts of $^1H\alpha$ and $^{13}C\alpha$ are plotted versus the residue number of the peptide in Figure 1B,C. As expected for α-helical regions (Wishart et al., 1991; Spera & Bax,

[2] CD spectroscopy of the peptide in TFE only showed a weak double minimum spectrum (θ at 222 and 208 nm), which is characteristic for α-helices. This means that the helix is not very stable or regular and is probably best described as a "nascent" helix (Dyson & Wright, 1991).

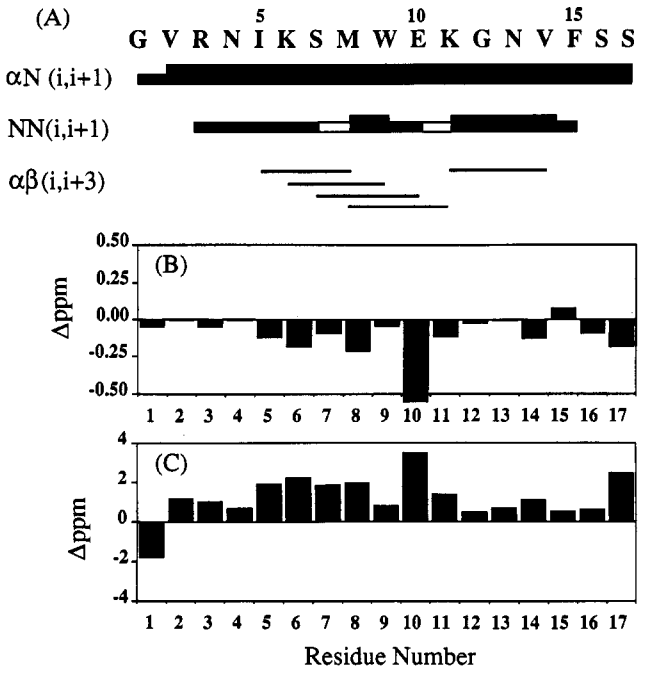

FIGURE 1: (A) Summary of NOE information obtained for the CaD peptide in 75% H_2O/25% TFE-*d* at pH 5.0. The thickness of the lines indicates the intensities of the NOE cross-peaks, which are strong (▬), medium (▬), and weak (▬). The open boxes represent the NOEs which presumably exist but were not observed due to the overlap of the resonances. (B and C) The secondary chemical shifts for $^1H\alpha$ (B) and $^{13}C\alpha$ (C) of the CaD peptide in 75% H_2O/25% TFE at pH 5.0. The random coil shifts of the αH and αC were taken from Wüthrich (1986) and Richarz and Wüthrich (1979), respectively.

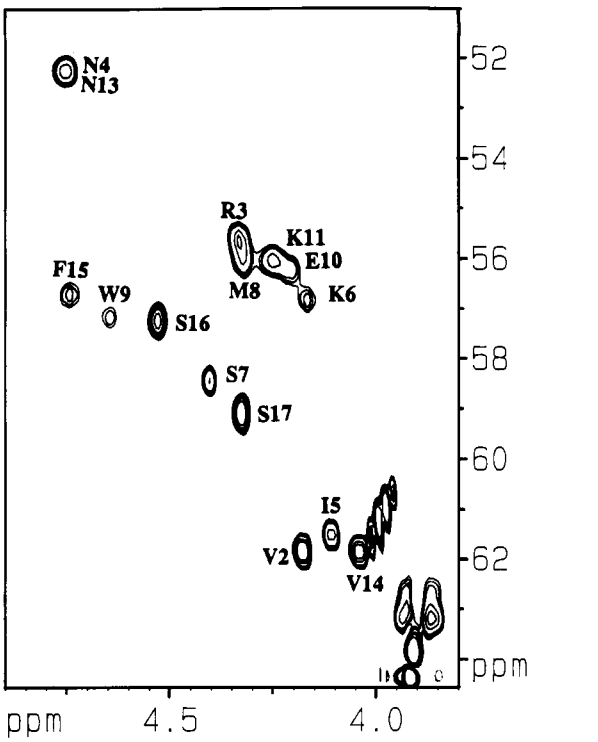

FIGURE 2: αCH region (^1H,^{13}C)-HMQC spectrum of the CaD peptide in aqueous TFE at pH 5.0. The assignment of the resonances is indicated by their residue name and number. The spectrum was recorded using a 5 mM peptide sample in 75% D_2O/25% TFE-*d*; 256 experiments with 128 scans per experiment were collected using a total of ≈20-h instrumental time. The sweep widths in F1 and F2 are 80 and 10 ppm, respectively, with the ^{13}C carrier centered at 30 ppm (relative to TSP at −1.6 ppm).

1991), most of the αH resonances in the central part of the peptide shift in the upfield direction, while all the $^{13}C\alpha$ shifts of the helical part of the peptide move downfield. The pattern of NOE connectivities combined with the secondary chemical shifts of the peptide clearly indicate that the central portion of the peptide can adopt a "nascent" α-helical structure.

The CaD Peptide Binds to CaM as an Amphiphilic α-Helix. The major goal of this study is to determine the structure of the CaD peptide when it is bound to CaM. Because of the relatively weak interaction, the transferred NOE experiment seems to be the most suitable technique for determining the structure of the peptide in its CaM bound form. In order to ascertain that CaM remains saturated with Ca^{2+} ions, a pH value of 6.5 was chosen for the TRNOE studies of the interaction between CaM and the CaD peptide. Figure 3 shows the aromatic region of the ^1H NMR spectra of CaD peptide in the absence and in the presence of a small amount of CaM. The addition of CaM at a ratio of 1:30 (protein: peptide) causes a severe line broadening of all the resonances as well as a shift in some of them. This result suggests that the peptide is in fast to intermediate exchange and that a significant amount of magnetization is transferred from the CaD peptide in the CaM bound form to the free form in the solution. A similar protein:peptide ratio has been used in other TRNOE studies of the binding of peptides to proteins (Landry & Gierasch, 1991; Campbell & Sykes, 1991). Subsequently, 2D ^1H NMR studies were carried out to further characterize the peptide structure in the CaM bound form.

NOESY spectra of the peptide/CaM complex show a lot more cross-peaks than the free peptide, this is the result of the longer correlation time of the bound peptide. The sequential assignment of the CaD peptide in the peptide/CaM complex at a ratio of 30:1 was obtained essentially by the same methods

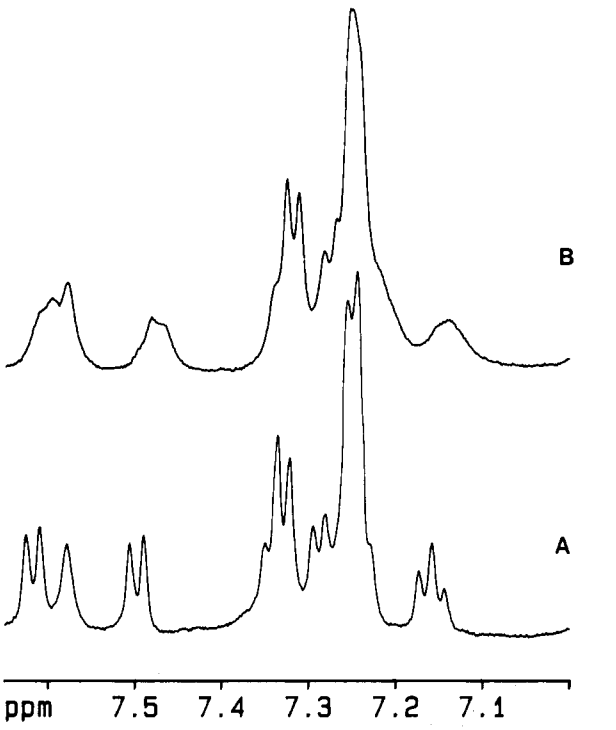

FIGURE 3: Aromatic region of the ^1H NMR spectra of (A) the free CaD peptide and (B) the CaD peptide/CaM mixture at a 30:1 ratio at pH 6.5. The line broadening and chemical shift changes that are induced by the addition of the small amount of CaM indicate that the free form and the CaM-bound form of the CaD peptide are in fast to intermediate exchange.

as used for the peptide in aqueous TFE. Table 1 provides the ^1H chemical shifts of all assigned residues of the peptide in

Table 1: ^{1}H Chemical Shifts of CaD Peptide in CaM/Peptide Mixture (1/30) at pH 6.5, 288 K[a]

	NH	αH	βH	others
G1	na[b]	3.76		
V2	na[b]	4.18	2.10	γCH_3 0.95, 0.95
R3	8.62	4.34	1.79	γCH_2 1.65, 1.58; δCH_2 3.17, 3.17
N4	8.64	4.72	3.00, 2.74	
I5	8.28	4.17	1.91	γCH 1.50, 1.20; γCH_3 0.89; δCH_3 0.90
K6	8.44	4.25	1.87, 1.75	γCH_2 1.44, 1.44; δCH_2 1.68; ωCH_2 2.97
S7	8.36	4.38	3.85, 3.77	
M8	8.41	4.39	2.00, 1.90	γCH_2 2.36
W9	8.06	4.69	3.35, 3.22	2H 7.24; 4H 7.61; 7H 7.48 5H 7.15; 6H 7.23
E10	8.09	4.20	2.10, 2.00	γCH_2 2.30
K11	8.30	4.18	1.78, 1.68	γCH_2 1.45; 1.45; δCH_2 1.68; ωCH_2 2.97
G12	8.41	3.95		
N13	8.30	4.71	2.74, 2.68	
V14	8.07	4.06	1.99	γCH_3 0.80
F15	8.41	4.72	3.18, 3.00	2,6H 7.30; 3,5H 7.36; 4H 7.13
S16	8.26	4.49	3.86, 3.86	
S17	8.02	4.27	3.88, 3.88	

[a] Chemical shifts are expressed in ppm relative to (trimethylsilyl)-propionic-d_4 acid (TSP) at 0 ppm. [b] Assignment not obtained.

the complex. Figure 4A shows the sequential assignment of the peptide in the fingerprint region. A substantial number of $d_{NN}(i,i+1)$ NOE cross-peaks were observed for the peptide in the complex (Figure 4B). Apart from the $d_{NN}(i,i+1)$ NOE cross-peaks, several weak $d_{\alpha N}(i,i+3)$ and $d_{\alpha\beta}(i,i+3)$ NOE cross-peaks were also detected in NOESY spectra of the peptide/CaM complex that were recorded in H_2O and D_2O, respectively (data not shown). The pattern of the NOE connectivities is summarized in Figure 5, and it clearly demonstrates that the CaM-bound peptide adopts an α-helical structure which runs from I5 to V14 in the peptide/CaM complex. Further analysis of this part of the peptide in a helical wheel diagram (Figure 6) shows that it has a characteristic amphiphilic structure with the hydrophobic residues on one side and the basic and hydrophilic residues on the other side of the helix.

CD spectroscopy has been used before to determine that peptides bind in an α-helical conformation to CaM (Cox et al., 1985; Erickson-Viitanen & DeGrado, 1987; Zhang & Vogel, 1994). We have compared the CD spectra of Ca^{2+}–CaM and the Ca^{2+}–CaM/CaD peptide complex, the latter has a slight increase in α-helicity over the former, suggesting that the peptide binds to CaM in a partially α-helical structure. No increase in α-helicity was observed in the CD spectra when the CaD peptide was added to apo-CaM in the presence of EDTA. Likewise, addition of apo-CaM to the CaD peptide in the presence of EDTA induced no broadening or shifts in the ^{1}H NMR spectrum of the CaD peptide. These data show that the interaction is calcium dependent.

The CaD Peptide Binds to Both Domains of CaM. Several experimental approaches were employed to study the interaction between CaM and the CaD peptide. Figure 7 displays a stackplot of the aromatic region of ^{1}H NMR spectra of Ca^{2+}–CaM during a titration of the protein with the CaD peptide. The resonances of a number of aromatic residues (e.g., Tyr138) are relatively sharp at the starting point of the titration, they broaden somewhat at the midpoint of the titration, and then they sharpen again at the end of titration; these resonances also experience a smooth chemical shift change throughout the titration. These results indicate that the CaM/peptide complex exists in a fast to intermediate exchange equilibrium between the protein-bound and free forms. Approximately 1.1 equiv of the peptide is needed to saturate Ca^{2+}–CaM. Higher concentrations of the peptide

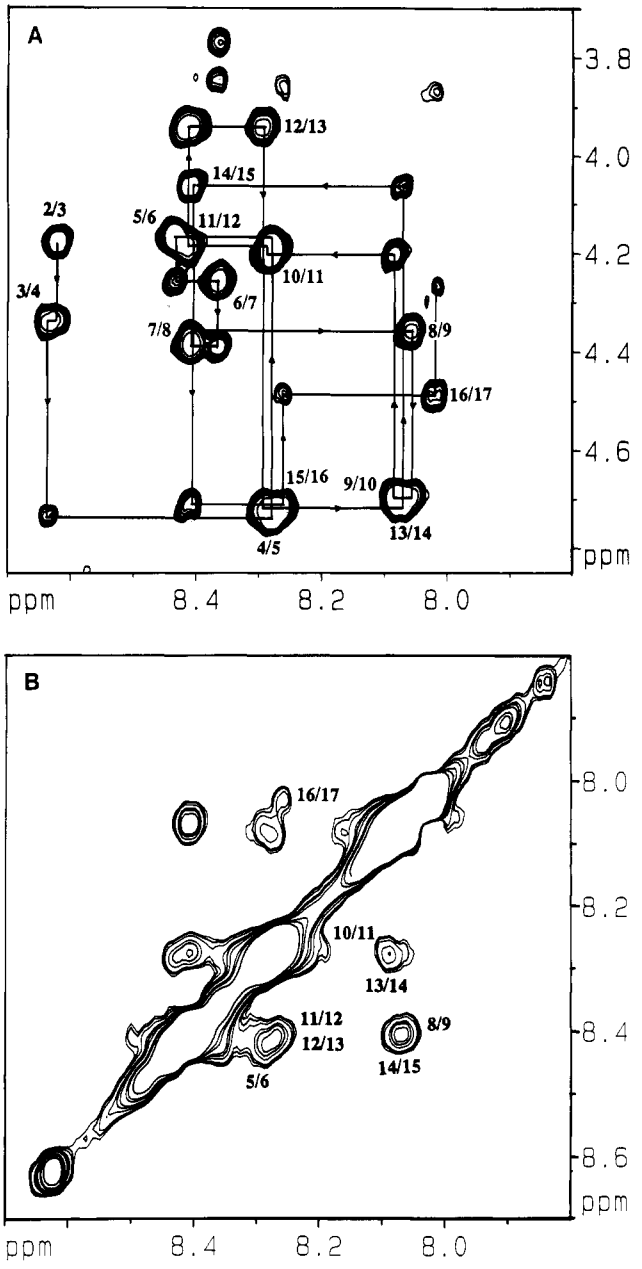

FIGURE 4: 200 ms NOESY spectrum of the CaD peptide/CaM mixture at 30:1 molar ratio at pH 6.5. (A) Fingerprint reguion of the NOESY spectrum showing the sequential assignment of the CaD peptide; (B) amide region of the same spectrum.

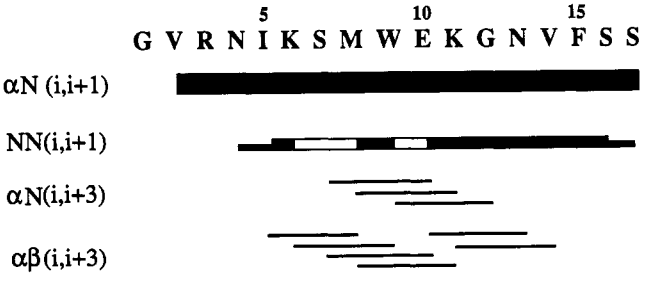

FIGURE 5: Summary of the NOEs observed for the CaD peptide from the TRNOE experiments of the peptide/CaM mixtures in H_2O and D_2O. The thickness of the lines indicates the relative intensities of the NOE cross-peaks as described in Figure 2.

did not cause further changes, from this we conclude that the peptide forms a 1:1 complex with CaM. As indicated in Figure 7, chemical shift changes were observed for Tyr138, F89, F99, F68, F16; this clearly indicates that both domains of

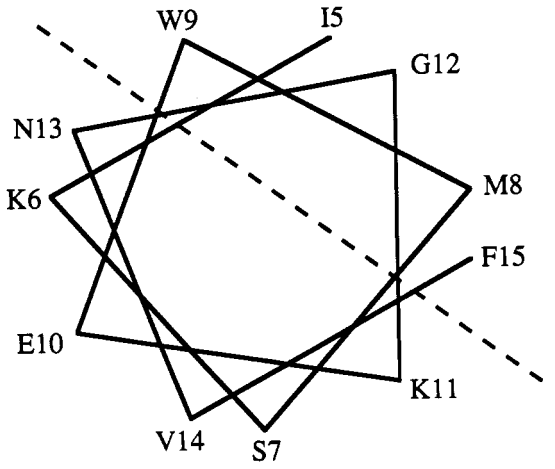

FIGURE 6: Helical wheel presentation of the α-helical part of the CaM-bound form of the CaD peptide. The helix shows clear amphiphilic properties; the hydrophobic and the hydrophilic face of the peptide are indicated by the broken line.

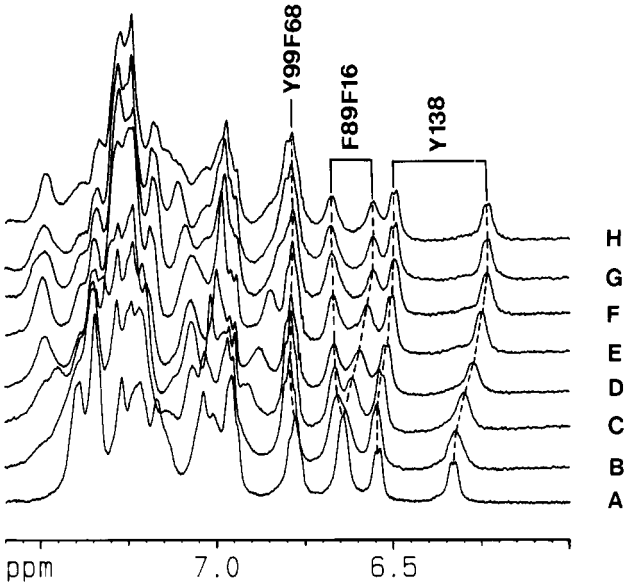

FIGURE 7: Stackplot of the aromatic region of the ^{1}H NMR spectra of CaM at different points of the titration with the CaD peptide (pH 7.0). The ratio of CaM:peptide is the following: (A) 1:0, (B) 1:0.2, (C) 1:0.4, (D) 1:0.6, (E) 1:0.8, (F) 1:1, (G) 1:1.1, and (H) 1:1.3. The assignment from several aromatic residues in CaM were previously obtained (Ikura et al., 1983, 1985) and are indicated in the figure.

CaM are involved in the binding of the CaD peptide. These results make it likely that both domains interact simultaneously with the peptide.

We have also studied a CaM sample, which was selectively labeled with ^{13}C-methyl groups on the Met residues. This allowed us to study the participation of the Met side chains in CaM in the binding of the CaD peptide. A titration of the ^{13}C-labeled CaM with the peptide was carried out, and Figure 8 shows some of the results obtained at various points of the titration. In free Ca^{2+}–CaM, the nine Met residues give rise to nine sharp well-resolved peaks. The assignment of resonances in Figure 8A was obtained by site-directed mutagenesis of the individual Met to Leu; the details of this experiment will be presented elsewhere (Zhang, Siivari, and Vogel, manuscript in preparation).[3] We collected a total of 10 spectra at various level of saturation (0–1.1). Because of the fast to intermediate exchange, it was possible to follow the changes of all the nine resonances during the titration. Thus, we have also been able to assign the resonances of the Met methyl groups of CaM in the CaM/peptide complex by following the peaks during the titration; this assignment is indicated in Figure 8D. Except for Met76, the rest of the eight Met resonances undergo severe line broadening and significant chemical shift changes. The line broadening probably arises from exchange and from the restricted motion of the Met methyl groups in the two domains of CaM upon binding the CaD peptide. The result suggests that at least eight out of nine Met residues are involved in the binding of the CaD peptide to CaM. Only Met76, which is a solvent-exposed residue on the linker region of CaM, remains in a similar orientation in the complex. We have attempted to study the contacts between the CaD peptide and the ^{13}C-methyl-Met groups of CaM directly through isotope-filtered NOE experiments (Roth et al., 1991; Ikura et al., 1992; Ikura & Bax, 1992). Unfortunately, no NOE cross peaks were observed. We believe that the broad lines of the methyl groups of the Met residues in the CaM/CaD peptide complex have prevented us from mapping out the orientation of the peptide in the complex in this fashion. Titration of ^{13}C-methyl-Met CaM with a peptide encompassing the CaM-binding domain of MLCK gives rise to a dispersion of the Met residues [data not shown, see also Ikura et al. (1992)] very similar to what is observed for the CaD peptide. This makes it extremely likely that the CaD peptide binds directly to the Met-rich regions of CaM, since it is known that the MLCK peptides do (Ikura et al., 1991; Meador et al., 1992).

The interaction of CaM with the peptide was further studied with ^{15}N uniformly labeled CaM. The backbone amide ^{15}N resonances of Ca^{2+}–CaM have already been assigned by Ikura et al. (1990); these assignements could be directly identified in our spectra. We have compared the (^{1}H,^{15}N)-HSQC spectra of ^{15}N uniformly labeled Ca^{2+}–CaM and Ca^{2+}–CaM/peptide complex (see Figure 9). A significant amount of backbone amide resonances from both domains undergo small chemical shift changes, and these amides include residues from loops and helices of the Ca^{2+}-binding motifs, as well as from the central α-helix. Again, these results support the notion that both domains of CaM are involved in the binding of the CaD peptide. Moreover, it indicates that the backbone structure of the central helix of the protein also undergoes conformational changes when the CaM/peptide complex is formed.

DISCUSSION

The binding of CaM to CaD is known to be quite different from the binding of this calcium regulatory protein to other target proteins. In particular, the interaction is almost ≈1000-fold weaker (Zhan et al., 1991), and hence it is unlikely that the complex of the CaM-binding domain of caldesmon with CaM will closely resemble that the MLCK (Ikura et al., 1992; Meador et al., 1992) or those of other proteins such as nitric oxide synthase (Zhang & Vogel, 1994) which have CaM-binding domains that share sequence homology with MLCK. Our CD and 2D ^{1}H NMR results obtained with the peptide in aqueous solution show that the 17-residue CaD peptide does not fold into a regular helical or turn structure under these conditions; this result is similar to what has been observed for the CaM-binding domain of MLCK in aqueous solution (Ikura & Bax, 1992; Zhang et al., 1993), and it is quite normal for a linear peptide of this size (Dyson & Wright, 1991).

[3] The assignment obtained in this way for the Met residues is almost identical as the one reported earlier by Ikura et al. (1991). However, the assignment for two Met residues, M124 and M144, has been interchanged.

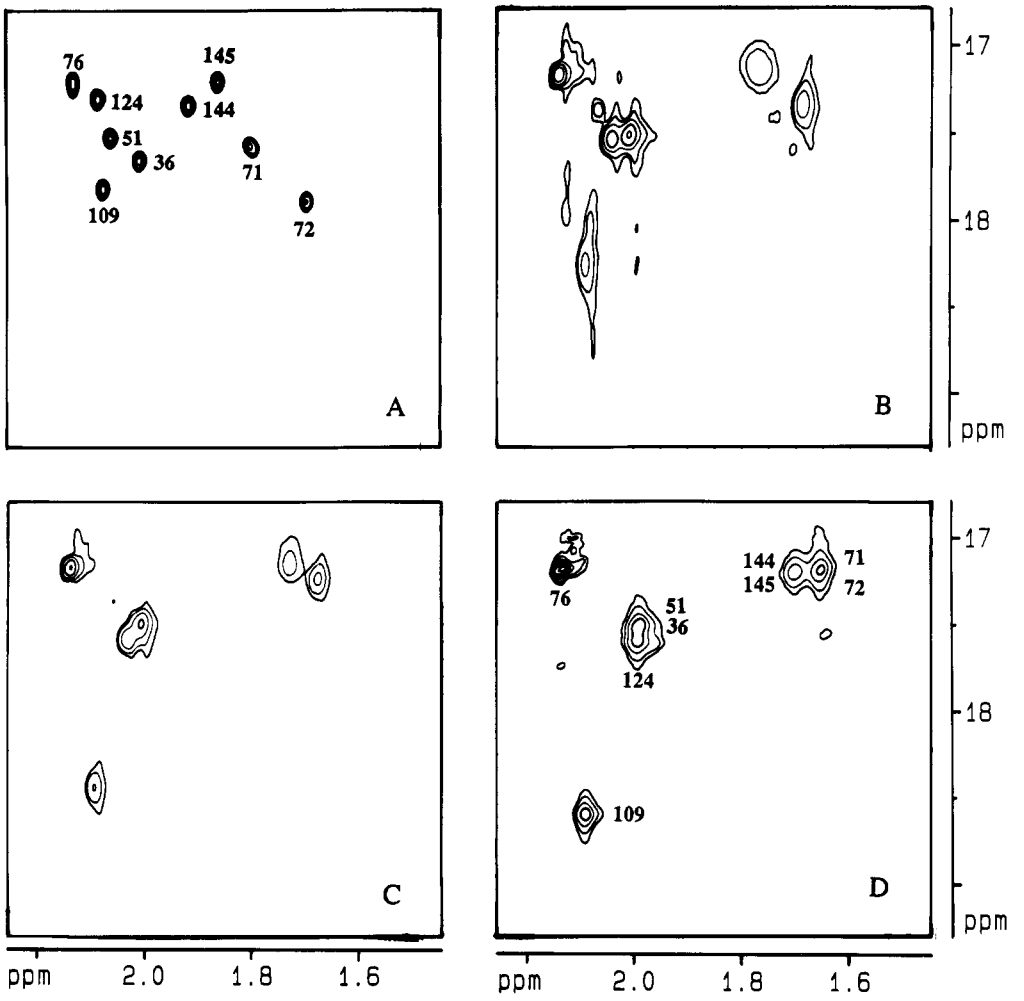

FIGURE 8: (^{1}H,^{13}C)-HMQC spectra of ^{13}C-methyl-Met selectively labeled CaM at various points during the titration with the CaD peptide. The ratio of CaM:peptide is (A) 1:0, (B) 1:0.4, (C) 1:0.7, and (D) 1:1.1. The salt concentration was 0.1 M KCl, the pH was 7.0, and the concentration of Ca^{2+}–CaM was ≈0.8 mM.

However, as indicated by the NOE pattern and the secondary chemical shifts (see Figure 1), the addition of the helix-promoting solvent TFE induces a short nascent α-helix in the central part of the peptide from residue I5 to V14. TFE is generally capable of inducing α-helical structure in those parts of a peptide that have helix-forming propensity (Nelson & Kallenbach, 1989; Lehnman et al., 1990; Dyson et al., 1992).

The relatively weak binding of the CaD peptide to CaM causes the NMR resonances for the free peptide and the protein complex to be in fast to intermediate exchange on the proton and carbon-13 NMR time scale (Figures 7 and 8). Unfortunately, the use of the elegant isotope filtering techniques that have been used to determine the structure of the bound MLCK CaM-binding domain peptide by NMR (Roth et al., 1991; Ikura & Bax, 1992; Ikura et al., 1992) was unsuccessful. However, when the exchange between the free and the bound peptide is fast relative to the spin-lattice relaxation time and the mixing time in the NOESY spectrum, the TRNOE experiment provides an alternate means to obtain information about the structure of the protein-bound peptide (Meyer et al., 1988; Milen et al., 1990; Ni et al., 1990; Campbell & Sykes, 1991; Landry & Gierasch, 1991). The appearance of the short range $d_{NN}(i,i+1)$ NOEs as well as the medium range NOEs shows that the CaM-bound peptide forms an α-helical structure from residue I5 to V14 (see Figure 5). The presence of α-helix in the bound peptide was further substantiated by CD spectroscopy of the complex. Interestingly, TFE induced α-helical turns in the same region of the CaD peptide in aqueous solution (see Figure 1). A similar result was obtained for a longer peptide encompassing the CaM-binding domain of MLCK (Zhang et al., 1993), suggesting that CaM and TFE share the capacity to induce amphiphilic α-helical structures in extended linear peptides that have helix-forming propensities.

The α-helix of the CaD peptide extends only over 10 residues, this is significantly shorter than the helix of the bound MLCK CaM-binding domain which extends over ≈18 residues (Roth et al., 1991; Ikura & Bax, 1992). Because of the lack of amino acid homology and the substantial difference in length, it was important to investigate the location of the CaD peptide binding site on CaM. The ^{1}H and ^{15}N NMR data presented in Figures 7 and 9 show that both domains of the protein are involved, and the ^{15}N backbone resonance of the linker region of CaM is influenced by the binding of the CaD peptide. In this respect, it is of interest that the side chain of Met76, which is also in the linker region of CaM, appears not to be influenced, whereas the so-called "methionine puddles" (O'Neill & DeGrado, 1990) in both domains of CaM are very likely involved in the binding of the CaD peptide (See Figure 8). In fact, the involvement of the methionine-rich regions has also been noted for the binding of the MLCK CaM-binding domain. These two areas are juxtaposed to the hydrophobic areas on the MLCK peptide; this arrangement is made possible by an unraveling of the α-helical linker region of CaM into a loop structure (Ikura et al., 1992). Thus, the most likely interpretation of our results is that the two

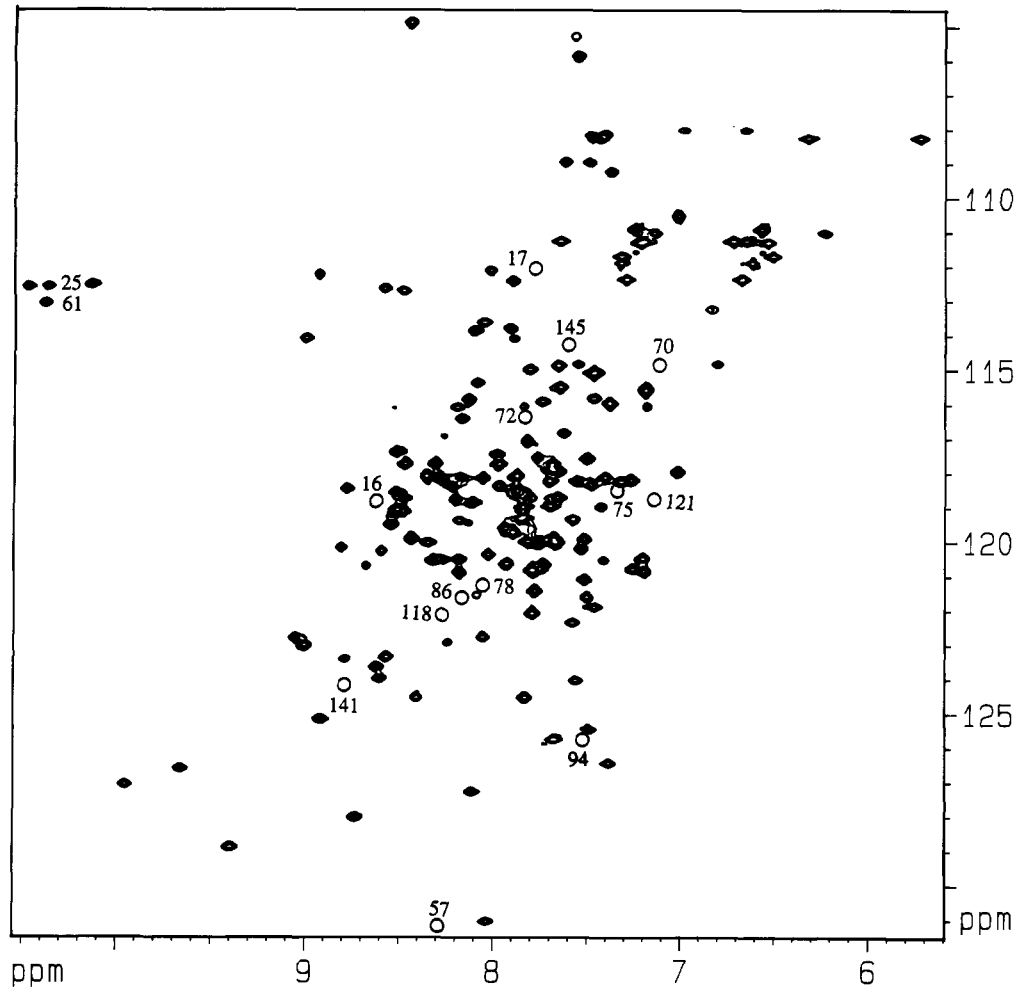

FIGURE 9: (^{1}H,^{15}N)-HSQC spectrum of the CaD peptide/CaM complex (1.1:1 molar ratio) in 0.1 M KCl, 25 °C, pH 6.5. The open circles represent the cross-peaks for some of the assigned amide resonances in CaM (Ikura et al., 1990). Clearly these resonances shift away upon complexation of the CaD peptide.

methionine-rich hydrophobic surfaces of CaM both bind to the hydrophobic face of the amphiphilic α-helical CaD peptide depicted in Figure 6. Our data do not allow us to conclude whether the two ends of the 17-residue CaD peptide bind to CaM as well. However, it is clear from the NOE patterns that they bind with an extended structure if they are directly involved in the interaction. Be that as it may, the orientation of the two domains of CaM in the complex with the CaD peptide would have to be different from that in the complex with the MLCK peptide, thus also requiring a different loop structure for the linker region of CaM. This may explain the lower affinity of CaM for CaD. Perhaps, the unique capacity of CaM to bind a wide range of hydrophobic/basic peptides arises from the flexibility of the central linker/loop structure, which could give rise to many different orientations of the two domains of CaM. This would allow them to interact with differently oriented hydrophobic patches on α-helical structures of different length.

ACKNOWLEDGMENT

We are indebted to Dr. T. Grundström, University of Umeå, for providing the synthetic calmodulin gene.

SUPPLEMENTARY MATERIAL AVAILABLE

Four figures and one table showing the sequential assignment and the chemical shift values of the CaD peptide in 25% TFE-*d* aqueous solution, a (^{1}H,^{15}N)HSQC NMR spectrum of ^{15}N-labeled CaM, and CD spectra of calmodulin and the calmodulin/peptide complex in the presence and absence of Ca^{2+} (8 pages). Ordering information is given on any current masthead page.

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