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A peptide analog of the calmodulin-binding domain of myosin light chain kinase adopts an α-helical structure in aqueous trifluoroethanol

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Abstract

A 22-residue synthetic peptide encompassing the calmodulin (CaM)-binding domain of skeletal muscle myosin light chain kinase was studied by two-dimensional NMR and CD spectroscopy. In water the peptide does not form any regular structure; however, addition of the helix-inducing solvent trifluoroethanol (TFE) causes it to form an α-helical structure. The proton NMR spectra of this peptide in 25% and 40% TFE were assigned by double quantum-filtered J-correlated spectroscopy, total correlation spectroscopy, and nuclear Overhauser effect correlated spectroscopy spectra. In addition, the α-carbon chemical shifts were obtained from (^{1}H,^{13}C)-heteronuclear multiple quantum coherence spectra. The presence of numerous dNN(i, i + 1), dαN(i, i + 3), and dαβ(i, i + 3) NOE crosspeaks indicates that an α-helix can be formed from residues 3 to 20; this is further supported by the CD data. Upfield α-proton and downfield α-carbon shifts in this region of the peptide provide further support for the formation of an α-helix. The helix induced by TFE appears to be similar to that formed upon binding of the peptide to CaM.

Keywords: α-helix; calmodulin; calmodulin-binding domain; CD; myosin light chain kinase peptide; NMR

Calmodulin is a ubiquitous regulatory calcium-binding protein that is present in all eukaryotic cells. The protein has an unusual dumbbell shape, consisting of two independently folded domains connected by a long α-helical linker (Babu et al., 1988). It can bind a total of four calcium ions in a partially positive cooperative fashion (Andersson et al., 1983; Thulin et al., 1984; Forsén et al., 1986). This allows CaM to undergo a substantial conformational change over a narrow range of intracellular Ca^{2+} concentrations (10^{-7}–10^{-6} M); this property makes it ideally suited as a regulatory switch (Hiraoki & Vogel, 1987). A large part of the calcium-induced conformational change involves the exposure of two hydrophobic surfaces (La Porte et al., 1980; Tanaka & Hidaka, 1980; Vogel et al., 1983). Compared with other proteins, CaM has a high number of Met residues and these are virtually all located in the hydrophobic surfaces (Babu et al., 1988). The two domains of CaM each contain one hydrophobic methionine-rich surface patch and two calcium-binding sites (Forsén et al., 1986; Hiraoki & Vogel, 1987).

The calcium-saturated form of CaM is capable of binding and activating almost 30 different target proteins such as myosin light chain kinase, cyclic nucleotide phosphodiesterase, CaM-kinases, calcineurin, caldesmon, etc. (for review see Means et al. [1991]). The CaM-binding domains of most of these proteins are contained in contiguous 20-residue peptides (Means et al., 1991). Recently the structures of complexes of Ca^{2+}–CaM with peptides containing the CaM-binding domains of skeletal and smooth muscle MLCK have been determined by NMR and X-ray methods (Ikura et al., 1992; Meador et al., 1992). These studies have revealed that the bound MLCK peptides adopt an amphiphilic helical structure, with its hydrophobic surfaces juxtaposed to the two hydrophobic patches on CaM. This mode of binding is consistent with earlier NMR (Seeholzer & Wand, 1989; Ikura et al., 1991; Roth et al., 1992), spectroscopic, and chemical modification studies (Cox et al., 1985; O'Neil & DeGrado,

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Abbreviations: CaM, calmodulin; DQF-COSY, double quantum-filtered J-correlated spectroscopy; HMQC, heteronuclear multiple quantum coherence; HPLC, high-pressure liquid chromatography; NOE, nuclear Overhauser effect; NOESY, NOE-correlated spectroscopy; MLCK, myosin light chain kinase; TFE, trifluoroethanol; TOCSY, total correlation spectroscopy; TPPI, time-proportional phase increment.

1990) of the complex. However, computer modeling of the interaction of the CaM-binding domain (residue K1 to R16 of the MLCK peptide studied in this work) with the remainder of the MLCK suggests that this part of the protein forms an extended structure rather than an α-helix (Knighton et al., 1992). This raises the intriguing possibility that the CaM-binding domain may actually have different conformations for its two protein-bound states.

In this work we have studied the structure of a 22-residue peptide analog of the MLCK CaM-binding domain (Blumenthal et al., 1985) in aqueous TFE. Our objective was to compare the peptide structure that can be induced in solution to that for the peptide bound to CaM. The solvent TFE was chosen because in pure H_2O the peptide does not fold into regular secondary structure. In addition, TFE is known to stabilize the formation of α-helices in peptides with helix-forming propensities (Nelson & Kallenbach, 1989; Lehnman et al., 1990; Segawa et al., 1991; Dyson et al., 1992; Sönnichsen et al., 1992; Zagorski & Barrow, 1992), but it has been shown recently that it is selective and that it generally does not do so in regions that are normally unstructured in proteins (Segawa et al., 1991; Dyson et al., 1992; Sönnichsen et al., 1992). Given this selectivity in inducing helical structure, aqueous TFE seems ideally suited for studying peptides that can bind to CaM because these are believed to comprise mainly α-helical regions (O'Neil & DeGrado, 1990). The CaM-binding domain of MLCK represents the only CaM-dependent target protein for which the structure of the CaM-bound form has been reported to date. Hence, at present, only for this peptide is it possible to compare directly the CaM-bound structure with those that can exist in solution.

Results

The 22-residue synthetic peptide studied here corresponds to the amino acid sequence of amino acid residues 577–598 in skeletal muscle MLCK; this region constitutes the CaM-binding domain of the protein (Blumenthal et al., 1985; Ikura et al., 1992). The one-dimensional ^1H NMR spectra of the peptide recorded in pure H_2O over a range of pH and temperature values showed little chemical shift dispersion in the amide region. Further two-dimensional NMR studies of the peptide also showed that the peptide is mainly in extended conformations in aqueous solution; this was judged from the absence of NH-NH crosspeaks in NOESY spectra, for example. The addition of different concentrations of TFE to the MLCK peptide induced a significant line broadening as well as chemical shift changes (Fig. 1), suggesting that the peptide undergoes a major structural change due to the change in environment. The line width of the MLCK peptide in TFE solutions was independent of the concentration of the peptide, suggesting that there is no association of the peptide in solution. The general appearance of the two-dimensional NMR spectra obtained in 25% TFE was slightly better

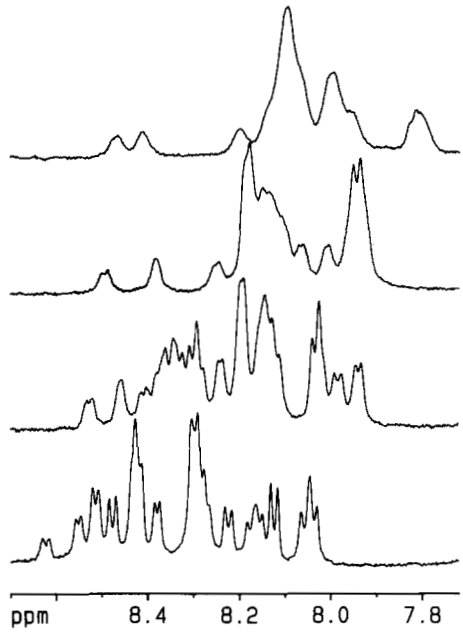

Fig. 1. The 500-MHz ^1H NMR spectra of the MLCK peptide at different concentrations (v/v) of TFE at pH 5.0, 288 K. The TFE concentrations, from bottom to top, are 0%, 15%, 25%, and 40%, respectively.

than those recorded in 40% TFE. Moreover, CD spectra (see below) showed the presence of a substantial amount of α-helix in 25% TFE. Consequently, the majority of the NMR work was done in this solvent. A temperature of 288 K was chosen for all NMR experiments (Fig. 2). At this temperature the resonance overlap of the CαH with the residual water (HDO) resonance is minimal. However, the NOE pattern for the peptide obtained at 298 K shows qualitatively the same picture as that obtained at 288 K (data not shown).

The sequential assignment of the peptide was obtained by following standard techniques for homonuclear ^1H two-dimensional NMR (Wüthrich, 1986). The DQF-COSY and TOCSY spectra of the peptide in the 75% H_2O/25% TFE mixture were used to identify spin systems. Subsequently, the sequence-specific assignment was traced out in the NOESY spectrum recorded in the same solvent with a mixing time of 200 ms (Fig. 2A). Table 1 provides the complete assignment of the peptide. Figure 2A shows that all sequential $d\alpha N(i, i + 1)$ NOEs and some medium-range $d\alpha N(i, i + 3)$ connectivities could be detected. Figure 2B displays the NH-NH region of the NOESY spectrum and reveals a large number of NH-NH connectivities. The pattern of the NOEs observed for the peptide is summarized in Figure 3. Fairly strong $dNN(i, i + 1)$ and $d\beta N(i, i + 1)$ NOEs were observed for residues from R3 to I20, although some gaps resulted from resonance overlap. Analysis of the NOESY spectrum of the peptide in the 75% D_2O/25% TFE mixture (data not shown) revealed a substantial number of $d\alpha\beta(i, i + 3)$

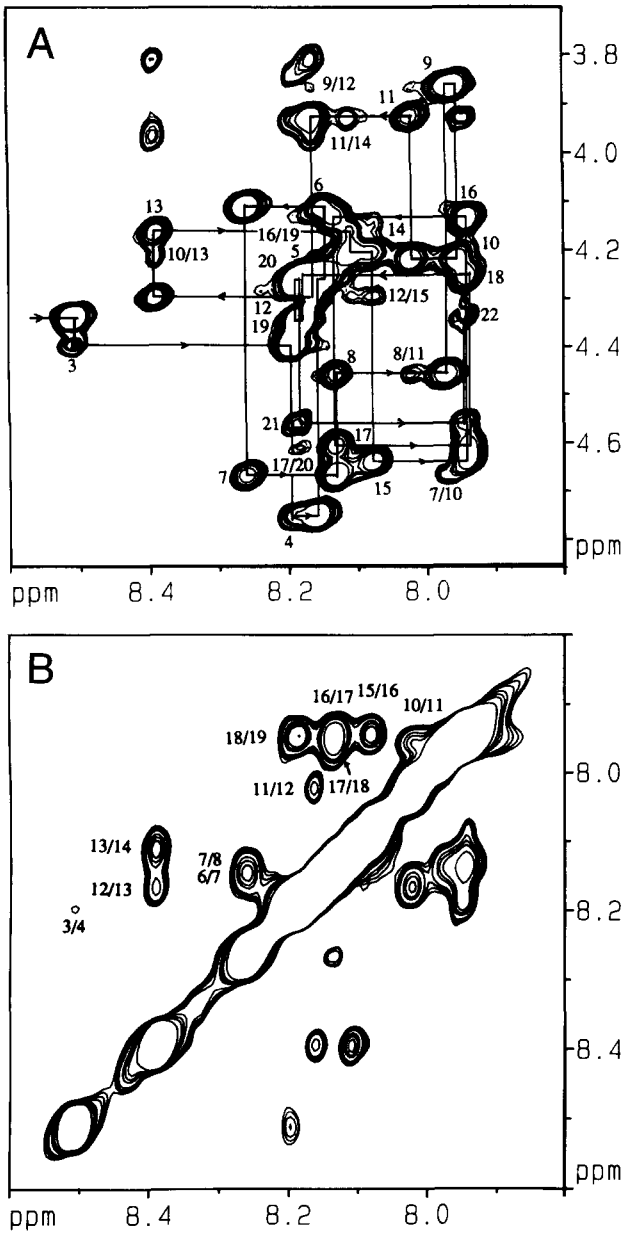

Fig. 2. A: Fingerprint region of the NOESY spectrum (200 ms). The sequential assignment of the NH-CαH resonance is indicated by the amino acid numbers. The dαN(i, i + 3) connectivities are indicated as the peaks that are labeled with two numbers. **B:** Amide region of the NOESY spectrum (200 ms). The assignments of the NH-NH crosspeaks are highlighted in the figure.

Table 1. *^{1}H and $^{13}C_\alpha$ chemical shifts of the MLCK peptide in H_2O/TFE mixture (75%/25%) at pH 5.0, 288 K*[a]

	NH	αH	$^{13}C_\alpha$	βH	Others
K1	na[b]	4.03	54.61	1.92, 1.74	γH: 1.49, 1.49; δH: 1.74; ϵH: 3.05
R2	8.76	4.33	55.59	1.78, 1.71	γH: 1.60, 1.57; δH: 3.12, 3.12
R3	8.50	4.39	55.94	1.78, 1.71	γH: 1.62, 1.55; δH: 3.12, 3.12
W4	8.19	4.75	56.09	3.32, 3.23	2H: 7.22; 4H: 7.63; 5H: 7.12; 6H: 7.22; 7H: 7.48; NH: 10.11
K5	8.16	4.25	55.93	1.90, 1.72	γH: 1.49, 1.49; δH: 1.70; ϵH: 2.96
K6	8.15	4.10	56.48	1.82, 1.70	γH: 1.39, 1.38; δH: 1.68; ϵH: 2.98
N7	8.26	4.66	52.72	2.81, 2.81	
F8	8.13	4.45	58.46	3.11, 3.11	2,6H: 7.19; 3,5H: 7.31; 4H: 7.30
I9	7.97	3.86	61.91	1.88	γH: 1.55, 1.18; γCH_3: 0.89; δCH_3: 0.8
A10	7.95	4.23	53.06	1.46	
V11	8.02	3.92	63.90	2.09	γCH_3: 0.96, 1.03
S12	8.16	4.29	59.17	3.80, 3.92	
A13	8.38	4.15	53.35	1.49	
A14	8.10	4.20	53.27	1.48	
N15	8.08	4.63	53.54	2.84, 2.84	
R16	7.94	4.13	56.91	1.88, 1.80	γH: 1.71, 1.71; δH: 3.10, 3.10
F17	8.13	4.60	57.54	3.29, 3.11	2,6H: 7.29; 3,5H: 7.36; 4H: 7.30
K18	7.94	4.24	55.93	1.90, 1.72	γH: 1.49, 1.49; δH: 1.70; ϵH: 2.96
K19	8.18	4.33	55.59	1.87, 1.72	γH: 1.42, 1.42; δH: 1.54; ϵH: 3.03
I20	8.18	4.27	60.75	1.95	γH: 1.54, 1.18; γCH_3: 0.90; δCH_3: 1.00
S21	8.18	4.55	57.25	3.83, 3.92	
S22	7.94	4.31	59.25	3.92, 3.92	

[a] Chemical shifts are expressed in ppm relative to (trimethylsilyl)-propionic-d_4 acid (TSP) at 0 ppm for ^{1}H and −1.6 ppm for ^{13}C, which corresponds to 67.7 ppm for dioxane.
[b] Assignment not obtained.

connectivities; these are also summarized in Figure 3. The observation of dαN(i, i + 3) and d$\alpha\beta$(i, i + 3) medium-range connectivities provides an indication that α-helical turns can form in the peptide. Near-complete resonance assignment was also obtained for the 60% H_2O/40% TFE sample (data not shown). The same structural information could be deduced from its NOE pattern. However, the intensities of some of the crosspeaks was significantly different when compared to the spectrum obtained in 25% TFE. In particular, the intensities of most dNN(i, i + 1) crosspeaks increased from medium to strong, whereas the majority of the dαN(i, i + 1) crosspeaks decreased from strong to medium.

Because more and more chemical shift data for proteins have been reported recently, it has become possible to demonstrate that the ^{1}H α-proton and ^{13}C α-carbon chemical shift values display a strong correlation with the secondary structure of peptide chains in a protein (Spera & Bax, 1991; Wishart et al., 1991). In order to measure the $^{13}C\alpha$ chemical shifts values for the MLCK peptide, a natural abundance (^{1}H,^{13}C)-HMQC spectrum was recorded for the peptide in the 75% D_2O/25% TFE mixture. Figure 4 shows the CαH region of this spectrum; the chemical shift values derived from this figure for the

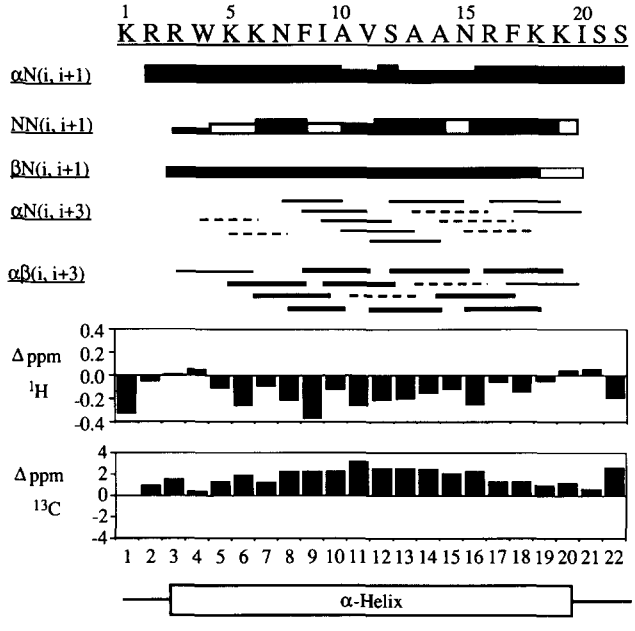

Fig. 3. Amino acid sequence from the peptide analog of the MLCK CaM-binding domain and a summary of the short- and medium-range NOEs involving the NH, CαH, and CβH protons. Some likely NOE crosspeaks that are presumably present but could not be detected because of resonance overlap are indicated by the open boxes and dashed lines. The thickness of the lines corresponds to the intensities of the measured NOEs. The secondary chemical shift of αH and αC of the MLCK peptide in 25% TFE are also presented. The random coil shifts of αH and αC were taken from Wüthrich (1986) and Richarz and Wüthrich (1978), respectively.

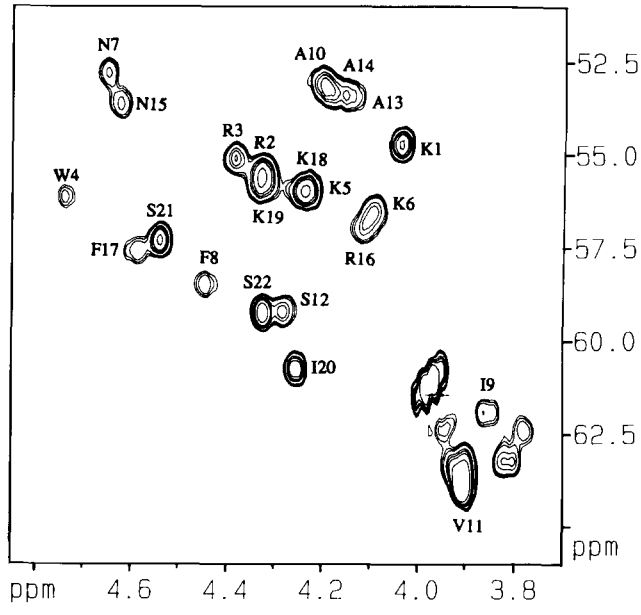

Fig. 4. Region of the HMQC spectrum, showing the assignment of the α-carbon resonances from the α-proton resonances.

α-carbons are listed in Table 1. A graphical representation of the secondary chemical shifts (the difference between the measured and random-coil chemical shifts) for both the ^{1}H α-protons and ^{13}C α-carbons of the peptide is given in Figure 3. As expected for an α-helical structure (Wishart et al., 1991), all αH resonances in the central part of the peptide shift in the upfield direction. Likewise, in agreement with previous reports (Spera & Bax, 1991; Wishart et al., 1991), all α-carbon shifts of the peptide are downfield. The maximum shifts observed for the peptide (−0.4 and +3.0 ppm, respectively) approximate the values measured for helical regions in intact folded proteins.

The temperature dependence of the amide proton chemical shift can provide further insight into the formation of secondary structure. An amide temperature coefficient of −8 to −11 ppb/K is normally observed for random coil peptides (Dyson et al., 1988), whereas α-helical regions generally display lower values (Deslauriers & Smith, 1980; Williamson & Waltho, 1992). For the 25% TFE/75% H_2O sample, the temperature dependence of all amide proton chemical shifts was linear in the range of 5–30 °C (data not shown). The terminal and penultimate residues of the MLCK peptide had values of −9 ppb/K, but the majority of the other amide protons had values of −5 to −6 ppb/K.

In order to further characterize the induction of α-helix in the MLCK peptide by TFE, we have used CD spectroscopy. The formation of a regular α-helix is generally accompanied by the appearance of two negative ellipticity minima at 208 and 222 nm (Chang et al., 1978; Johnson, 1990). However, unlike NMR, CD studies cannot show the specific regions of the peptide that are folded into a regular α-helical structure. The data in Figure 5 illustrate

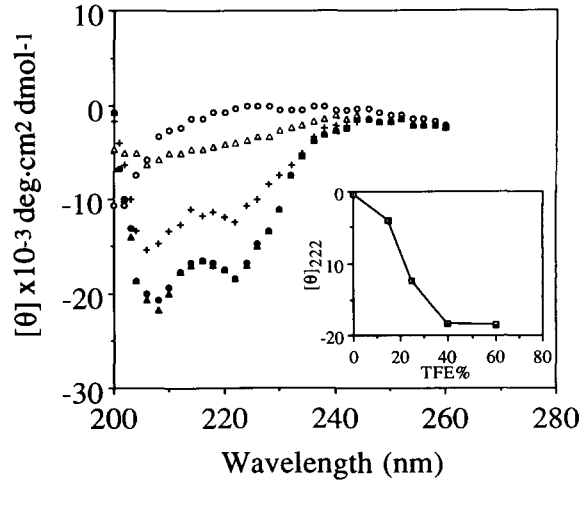

Fig. 5. UV-CD spectra obtained for the MLCK peptide under the following conditions: 0% TFE (○), 15% TFE (△), 25% TFE (+), 40% TFE (●), and 60% TFE (▲). The inset shows the ellipticity measured at 222 nm as a function of the concentration of TFE.

clearly the absence of regular secondary structure in 0% TFE, whereas at 25% and 40% TFE, a substantial amount of helix is formed (up to ~50%).

Discussion

In agreement with earlier reports (Seeholzer & Wand, 1989; Ikura & Bax, 1992), our NMR and CD data show that the 22-residue MLCK CaM-binding peptide does not adopt regular secondary structure in aqueous solution. However, upon addition of TFE, the diagnostic negative ellipticities at 208 and 222 nm, which are indicative of the formation of α-helix, are observed in the CD spectra. For the MLCK peptide in aqueous TFE, the backbone and side-chain proton NMR resonances could be completely assigned in a sequential manner with the aid of DQF-COSY, TOCSY, and NOESY spectra. The observation of dNN(i, $i+1$), dαN(i, $i+3$), and d$\alpha\beta$(i, $i+3$) crosspeaks in the NOESY spectra suggests clearly that the peptide can potentially adopt a continuous α-helical structure. Although some dNN(i, $i+1$) connectivities could not be established because of crosspeak overlap, redundancy in the data (Fig. 3) suggests that an α-helix can be formed from residue 3 to residue 20. Also, the somewhat lower values of temperature dependence of the amide proton chemical shifts in the central part of the peptide are consistent with α-helix formation, but even lower values are usually obtained for stable helices in proteins. Finally, some indication of the presence of an ordered helix is obtained from the upfield shift of the αH protons; a similar upfield shift is found for these protons in α-helical regions of intact proteins (Wishart et al., 1991). In addition, nearly all α-carbon atoms shift downfield, a trend that has also been observed previously for α-helices in folded proteins (Spera & Bax, 1991; Wishart et al., 1991). A Gaussian-type distribution for the ^{13}C and ^{1}H chemical shifts of αCH (Fig. 3) suggests that the MLCK peptide may have a more ordered α-helix in the center, and that the two ends experience fraying. However, it is known that co-solvents such as TFE can have an effect on the chemical shift of amide resonances (Llinas & Klein, 1975), hence the possibility that the αH and αC shifts are also affected to some extent by the solvent cannot be ruled out. For this reason, it is difficult to attach a detailed interpretation to the substantial shifts observed here.

Linear peptides rarely adopt one stable conformation in solution. Instead, they usually exist as an ensemble of conformations that are in rapid exchange on the NMR timescale (Williamson & Waltho, 1992). Dyson, Wright, and coworkers (1991, 1992) have proposed a scheme for the classification of helices and helical turns in linear peptides based on the observed NOE patterns and CD spectra. A significant population of conformations containing "ordered" α-helix is present when the characteristic double-minimum CD spectrum as well as the continuous short-range dNN(i, $i+1$) and medium-range dαN(i, $i+3$) and d$\alpha\beta$(i, $i+3$) NOE connectivities are observed. A conformational ensemble containing "nascent" helix is characterized by the absence of the diagnostic CD spectrum, detection of dNN(i, $i+1$), and sometimes d$\alpha\beta$(i, $i+3$) and/or dαN(i, $i+2$) NOE crosspeaks. Clearly our data show that we have a significant population of ordered α-helical structure for the MLCK peptide in aqueous TFE. However, the presence of strong dαN(i, $i+1$) connectivities in 25% TFE suggests that this structure is in equilibrium with extended structures. At 40% TFE, the altered dNN(i, $i+1$)/dαN(i, $i+1$) crosspeak intensity ratio indicates that the equilibrium is shifted further toward the ordered helix.

In the complex of the MLCK peptide with CaM, the helix extends from residue 3 to 21, whereas residues on either end appear to have no regular structure (Ikura & Bax, 1992; Ikura et al., 1992; Meador et al., 1992). The similarity of the helices induced by CaM and aqueous TFE suggests that studies with the latter solvent may provide some insight into the structures of the CaM-binding domain peptides of other target proteins. Indeed, NMR studies of a 17-residue CaM-binding peptide derived from caldesmon (Zhan et al., 1991) have shown that this peptide also adopts identical structures when bound to CaM and in aqueous TFE solutions (Zhang & Vogel, 1993), lending further credence to this notion. It is important to stress that these results do not imply that the induction of α-helical structure in the MLCK peptide by CaM or TFE follows the same mechanism. Although it is more elegant to determine the structure of the bound peptide directly in the complex with CaM by NMR, such studies generally require the availability of totally ^{13}C/^{15}N-labeled CaM (Ikura & Bax, 1992) or ^{15}N-labeled peptides (Roth et al., 1991). The cost involved in the isotope labeling can make this approach prohibitive, particularly when the structures of a series of peptides with amino acid substitutions are compared. Availability of TFE-induced "bound" peptide structures of other target proteins of CaM will facilitate molecular modeling of their peptide CaM complexes. A similar approach has been used to study the binding of melittin to CaM (Strynadka & James, 1990). Because the two domains of CaM are known to retain their conformation upon complex formation (Ikura et al., 1992; Meador et al., 1992; Roth et al., 1992), such docking studies with NMR-derived CaM-binding peptide structures may prove insightful.

The helical structure of the CaM-binding domain of MLCK is not observed in the structure proposed for this enzyme. The three-dimensional structure for MLCK was obtained by homology modeling to a related protein kinase (Knighton et al., 1992). In this model, the CaM-binding domain is in an extended conformation, which is stabilized by numerous electrostatic interactions. Given the good fit obtained, this model seems very reasonable. It could be argued that the modeled protein is from the smooth muscle system and hence should have no bearing

on results obtained with skeletal muscle protein. However, the CaM-binding domains of these two proteins can be interchanged with complete preservation of function (Leachman et al., 1992). Thus, CaM regulation appears to be controlled in an identical manner for these two proteins. Moreover, the structure of their CaM-bound target peptides is nearly identical (Ikura et al., 1992; Meador et al., 1992). Taken together, these data suggest that a major conformational change of the CaM-binding domain, which involves a reorganization of the secondary structure, is required for the activation of MLCKs by calcium-CaM.

Materials and methods

The peptide (KRRWKKNFIAVSAANRFKKISS) was synthesized by the Core Facility for Protein/DNA Chemistry at Queen's University (Kingston, Canada). Purity of the peptide was greater than 95% as judged by amino acid analysis and HPLC. The D_2O and deuterated TFE were obtained from MSD Isotopes (Montreal, Canada).

Two samples, one in 75% H_2O/25% TFE and the other in 75% D_2O/25% TFE, were used for the majority of the NMR studies. The concentration of the samples was about 5 mM; the pH was adjusted to 5.0 by addition of the proper amount of KOD or DCl; no corrections for isotope effect were made. A third sample containing 60% H_2O/40% TFE was also prepared with a peptide concentration of ~3 mM (pH 5.0).

The NMR spectra were recorded at 288 K on a Bruker AMX500 spectrometer equipped with a 5-mm inverse detection probe. All spectra were recorded in the pure phase absorption mode by using the TPPI method to obtain quadrature detection in F1 (Marion & Wüthrich, 1983). The TOCSY (Bax & Davis, 1985) and NOESY (Bodenhausen et al., 1984) spectra were recorded for all samples. A mixing time of 200 ms was used for NOESY experiments; mixing times of 55 and 75 ms were used for the TOCSY experiments. The DQF-COSY spectra (Rance et al., 1983) were acquired for the sample in H_2O/TFE mixture at five different temperatures ranging from 5 to 30 °C in order to obtain the temperature dependence of the amide proton chemical shifts. For the TOCSY, DQF-COSY, and NOESY spectra recorded in H_2O, the water resonance was suppressed by presaturation over a sweep width of ~25 Hz. The HMQC spectra of the peptide sample in D_2O/TFE were recorded using the method of Bax et al. (1983). All spectra were processed on an X32 computer using the Bruker UXNMR software package. All data were zero filled once in the F1 dimension, and a sine-square window function with a 60° phase shift was used during the Fourier transformation. The intensity of the crosspeaks were classified as weak, medium, and strong by counting contour levels in the NOESY spectra.

The CD spectra were obtained at 288 K on a Jasco J-500C spectropolarimeter using cells with a pathlength of 0.1 cm. The peptide concentration was 15 μM. The peptide was dissolved in a 5 mM citric acid buffer, pH 5.0.

Acknowledgments

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