Articles

Isotope-Edited Fourier Transform Infrared Spectroscopy Studies of Calmodulin's Interaction with Its Target Peptides[†]

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ABSTRACT: The ubiquitous calcium-binding protein calmodulin (CaM) regulates a wide variety of cellular events by binding to and activating many distinct target enzymes. The CaM-binding domains of most of these enzymes are contained in a contiguous stretch of amino acids with a length of ≈ 20 residues. In this work, we have used "isotope-edited" Fourier transform infrared spectroscopy to study the interaction of CaM with synthetic peptides resembling the CaM-binding domains of myosin light chain kinase (MLCK), constitutive nitric oxide synthase (cNOS), and caldesmon (CaD). Uniform labeling of CaM with carbon-13 causes the amide I band of the protein to shift ≈ 55 cm^{-1} to lower frequency in D_2O, leaving a clear window in the infrared spectrum for observing the amide I band of the unlabeled target peptides. Upon complex formation, the amide I bands of the CaM-binding domains of MLCK and cNOS shift 4 cm^{-1} toward higher frequency (to ≈ 1648 cm^{-1}), and have a narrower bandwidth compared to the peptide in aqueous solution. These spectral changes and the fact that the infrared spectra of these two peptides in their complex with CaM closely resemble those recorded in a mixture of D_2O and the helix inducing solvent trifluoroethanol indicate that they bind to CaM in an α-helical conformation. The CaM-binding domain of CaD also showed similar, but less dramatic, spectral changes; this is in agreement with the fact that it binds to CaM with lower affinity and a shorter α-helix. Binding of these three peptides to CaM gave rise to only minor changes in the secondary structure of CaM. The strategy described in this work, which utilizes uniformly isotopically ^{13}C-labeled CaM to shift the amide I band of the protein from the original spectral region, should prove useful for determining the secondary structure of CaM-binding domains of other target proteins. Moreover, it should be generally applicable in studies of other protein–protein, protein–peptide, and protein–nucleic acid interactions.

Calmodulin (CaM[1]) is a small, acidic calcium-binding protein which is present in all eukaryotes. The X-ray structure of Ca^{2+}–CaM shows that the protein has two globular domains which are connected by a long, solvent-exposed α-helix (Babu et al., 1988); NMR studies reveal that this α-helix is rather flexible in solution (Ikura et al., 1991; Barbato et al., 1992). The binding of two calcium ions to each globular domain induces significant conformational changes in the protein and exposes two Met-rich hydrophobic surfaces (one in each domain) through which the protein binds to its targets (Vogel et al., 1983; Hiraoki & Vogel, 1987). The Ca^{2+}-saturated form of CaM is capable of regulating a wide variety of cellular

events by binding to and activating almost 30 different target enzymes such as MLCK, cyclic nucleotide phosphodiesterase, and cNOS (for a review, see Means et al. (1991)). The CaM-binding domains of most of these proteins are contained in a continuous stretch of about 20 amino acid residues (Means et al., 1991; O'Neil & DeGrado, 1990). There is no obvious amino acid sequence homology between CaM-binding domains from various target enzymes, although these peptides do share a tendency to form basic amphiphilic α-helices. The CaM-bound structure of the CaM-binding domains of a few target enzymes have been determined by X-ray crystallography (Meador et al., 1992; 1993) and multidimensional heteronuclear NMR spectroscopy (Roth et al., 1991; Ikura & Bax, 1992; Ikura et al., 1992). However, such a complete structure determination by X-ray crystallography or NMR is not always feasible, and hence, there is still a need for alternative spectroscopic approaches.

CD spectroscopy has been frequently used to study the interaction between CaM and peptides (for example, see Malencik and Andersson (1984), Erickson-Viitanen and DeGrado (1987), and Zhang and Vogel (1994a)). The CD spectrum of CaM shows the protein to be primary α-helical; peptides which bind to CaM are generally devoid of such secondary structure in aqueous solution. However, an increase in α-helicity is observed when the two components are mixed. This result has been interpreted as suggesting that the peptide

binds with an α-helical structure to CaM (Malencik and Andersson, 1984; Erickson-Viitanen & DeGrado, 1987; Zhang & Vogel, 1994a). However, it is also possible that the α-helicity of CaM increases and that the peptide binds with an irregular conformation. While originally considered unlikely, this notion has gained some credibility when it was shown that the α-helical content of CaM increases upon addition of various secondary structure inducing solvents (Bayley & Martin, 1992). In order to resolve this issue, it will be necessary to study the secondary structure of the two components of the complex separately, but this cannot be done by CD spectroscopy because the α-helical CD bands of the two components overlap.

FTIR spectroscopy has emerged in the last decade as a valuable technique to quantitatively determine secondary structure formation in proteins by studying the position and intensity of the conformation-sensitive amide I band (Surewicz & Mantsch, 1988; Surewicz et al., 1993). In particular, the technique has shown great promise for the study of conformational changes in proteins (Casal et al., 1988; Prestrelski et al., 1991; Dong et al., 1992; Fabian et al., 1993). However, when two different proteins are present in solution, their amide I bands also overlap, making the interpretation ambiguous as with CD spectroscopy. It has been reported that substitution of the carbon-13 isotope in the carbonyl group causes a shift of 36–45 cm^{-1} of the amide I band (essentially a C=O stretching vibration of the amide carbonyl groups) to lower frequencies (Hübner et al., 1990; Tadesse et al., 1991; Harris et al., 1992). Hence, by mixing a totally isotopically carbon-13 labeled protein with an unlabeled protein or peptide, it should be feasible to study their respective amide I bands separately. Here we have used this strategy to study the interaction of CaM with synthetic peptides derived from the CaM-binding domain of three different target proteins, i.e., MLCK, cNOS, and CaD. The first two peptides bind with a $K_D \approx$ 1–2 nM to CaM, while the K_D for the third peptide $\approx 1 \mu$M. Previous NMR studies of these peptides have shown that they are largely devoid of regular secondary structure in aqueous solution; however, upon binding to CaM they adopt an α-helical conformation (Ikura & Bax, 1992, Zhang et al., 1993; Zhang & Vogel, 1994a,b). Our results will demonstrate that "isotope edited" infrared spectroscopy corroborates these results.

MATERIALS AND METHODS

All reagents used are analytical grade. The 22-residue MLCK peptide (KRRWKKNFIAVSAANRFKKISS), 23-residue cNOS peptide (KRRAIGFKKLAEAV KFSAK-LMGQ), and 17-residue CaD peptide (GVRNIKSM-WEKGNVFSS) were commercially synthesized and used as described previously (Zhang et al., 1993; Zhang & Vogel, 1994a,b). Unlabeled bovine CaM was overexpressed in and purified from *Escherichia coli* cells as described earlier (Zhang & Vogel, 1993). ^{15}N/^{13}C-uniformly labeled CaM was obtained in a manner identical to that for ^{15}N-uniformly labeled CaM (Zhang & Vogel, 1994b) except that ^{13}C$_6$-glucose (Isotec Inc., 1 g/L) rather than unlabeled glucose was used in the culture medium. ^{15}N/^{13}C-uniformly labeled CaM was purified using the same method as that used for the unlabeled protein. Apo-CaM was prepared by passing CaM though a Chelex-100 column equilibrated with 100 mM NH$_4$HCO$_3$, pH 8.0. For FTIR measurements in D$_2$O, apo-CaM was dissolved in 50 mM HEPES/D$_2$O buffer at 20 mg/mL, and the pH (direct readings from a pH meter) of the sample was adjusted to 7.5 by adding microliter amounts of diluted KOD or DCl. The apo-form of CaM was left overnight at room
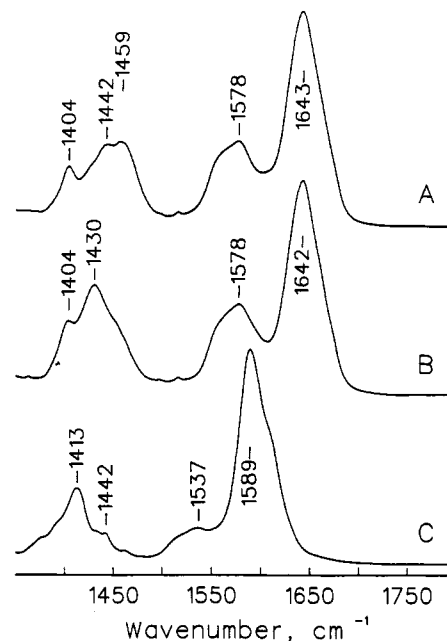

FIGURE 1: Amide I and amide II regions of the infrared spectra of (A) unlabeled, (B) ^{15}N-uniformly labeled, and (C) ^{13}C/^{15}N-uniformly labeled CaM in D$_2$O saturated with Ca^{2+}. The protein was dissolved in 50 mM HEPES/D$_2$O buffer at pH 7.5.

temperature to ensure the complete exchange of amide protons with deuterium. The Ca^{2+}-form of CaM was then prepared by adding the appropriate amount of CaCl$_2$ (0.5 M stock in D$_2$O) into the apo-CaM solution. This procedure was followed because hydrogen exchange is known to be faster in the apo protein than the Ca^{2+} protein. The protein and peptide concentrations used in this study were comparable to those used in our earlier NMR work; hence, we know that the appropriate protein/peptide complexes are formed under the experimental conditions used.

The FTIR spectra were measured on a Bio-Rad Digilab FTS-40A spectrometer equipped with a liquid nitrogen cooled MCT detector and continuously purged with dry air. The samples were placed between two CaF$_2$ windows separated by a path length of 45 μm at ambient temperature. To compensate for the D$_2$O absorption, the buffer solutions were measured in a cell with a slightly shorter path length. A total of 512 interferograms, with a resolution of 2 cm^{-1}, were collected for each spectrum. A boxcar apodization function was applied prior to the Fourier transformation. Subtractions of buffer spectra and corrections for water vapor were carried out in a similar manner as described previously (Fabian et al., 1992).

RESULTS

Figure 1 shows the infrared spectra of unlabeled, ^{15}N-uniformly labeled, and ^{13}C/^{15}N-uniformly labeled CaM in their Ca^{2+}-saturated state in D$_2$O, respectively. In agreement with an earlier study (Harris et al., 1992), ^{15}N-uniform labeling of CaM only causes subtle changes to the amide I band and to certain side chain bands of the infrared spectrum of CaM in D$_2$O (Figure 1A,B). Hence, the uniformly ^{15}N-labeled protein, which is comparatively cheap to prepare, does not offer much of an improvement compared to the unlabeled protein for the study of protein–protein or protein–peptide interactions. However, ^{15}N-uniform labeling causes a significant shift of the amide II band (N–H bending vibration strongly coupled to C–N stretching) of the protein in D$_2$O (from 1459 to 1430 cm^{-1}). Similarly, the amide II band of
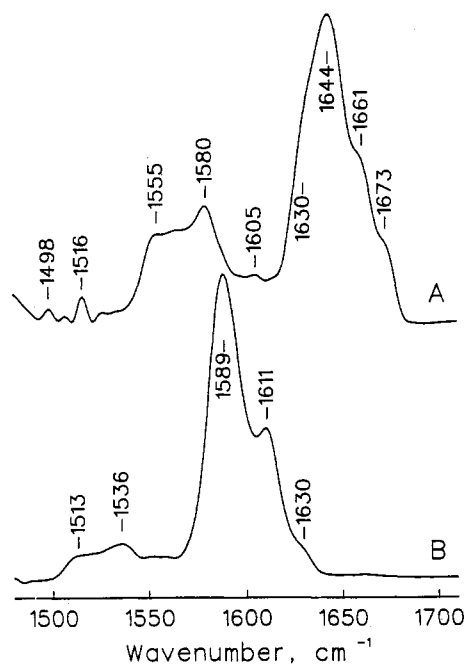
FIGURE 2: Deconvolved infrared spectra of (A) unlabeled and (B) uniformly $^{15}N/^{13}C$-labeled Ca^{2+}–CaM in 50 mM HEPES/D_2O. Band narrowing by Fourier self-deconvolution was performed as described previously (Moffatt & Mantsch, 1992) using a Lorentzian line shape with a half-bandwidth of 16 cm^{-1} and a resolution enhancement factor of 2.

FIGURE 3: Deconvolved infrared spectra of (A) $^{15}N/^{13}C$-labeled CaM (dashed line) and its complex with the MLCK peptide at 1:1 ratio (solid line), (B) $^{15}N/^{13}C$-labeled CaM (dashed line) and its complex with the cNOS peptide at 1:1 ratio (solid line), and (C) $^{15}N/^{13}C$-labeled CaM (dashed line) and its complex with the CaD peptide at 1:1 ratio (solid line). All spectra were recorded in 50 mM HEPES/D_2O buffer, pH 7.5, under identical conditions. The bands centered at 1674 cm^{-1} result from trifluoroacetate (a residual from the peptide synthesis). The bands centered at 1648 and 1646 cm^{-1} are the amide I band of the MLCK peptide and the cNOS peptide complexed with CaM, respectively. The CaD peptide has a lower intensity in the amide I region since this peptide is shorter and binds weaker to CaM compared to the other two peptides.

the protein in H_2O also shifted from 1551 to 1534 cm^{-1} as a result of ^{15}N-uniform labeling, and this frequency shift might be useful for studying deuterium–proton exchange of the protein in a complex with a peptide or another protein (spectra not shown). The spectral changes induced by uniform labeling with carbon-13 are more pronounced (Figure 1C). The maximum of the amide I band for unlabeled CaM in D_2O is centered at 1643 cm^{-1}. Uniform labeling of the protein with carbon-13 results in a 55 cm^{-1} shift of the amide I band toward lower wavenumber, and in doing so leaves a clear window in the spectrum between 1640 and 1700 cm^{-1}, which should make it possible to unambiguously observe the amide I band of an unlabeled protein or of a peptide when CaM is mixed with its targets. It should be noted that the absence of any remaining intensity for the amide I band at 1643 cm^{-1} indicates that the protein has been labeled for >99% with the carbon-13 isotope. This is significantly higher than reported in an earlier study (Harris et al., 1992); such a high incorporation of isotope is needed to study the relatively small amide I bands of the target peptides (*vide infra*).

Figure 2 shows the Fourier self-deconvoluted infrared spectra of unlabeled (Figure 2A) and $^{13}C/^{15}N$-uniformly labeled (Figure 2B) CaM in D_2O, respectively. The strongest band located at 1644 cm^{-1} in Figure 2A originates from the α-helices of the protein which account for $\approx 65\%$ of the total secondary structure components (Babu et al., 1988). The bands at 1661 and 1673 cm^{-1} are assigned to "turnlike" structure (Surewicz & Mantsch, 1988) present in CaM (Babu et al., 1988). The lack of pronounced infrared band(s) in the range of 1620–1640 cm^{-1}, even after band-narrowing by Fourier self-deconvolution, excludes the presence of a significant amount of β-sheet structure in CaM in aqueous solution. This is in good agreement with the X-ray data, where only two mini β-sheets formed by the Ca^{2+}-binding loops of the protein were found (Babu et al., 1988). Only 12 residues, or 8% of the whole molecule, are present in a β-sheet. This small amount of β-sheet structure, which is known to be present also in aqueous solution (Ikura et al., 1991), may be reflected
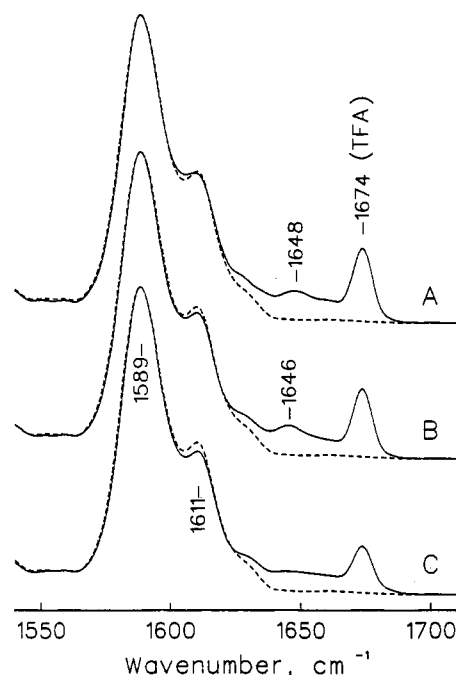
by the shoulder near 1630 cm^{-1} in Figure 2A. Bands between 1480 and 1610 cm^{-1} (in fully H–D exchanged proteins) are due to amino acid side chain absorption: Tyr (1516 cm^{-1}), Phe (1498 cm^{-1}), Arg (1605 cm^{-1}), and overlapping bands of Glu and Asp (between 1550 and 1580 cm^{-1}) (Chirgadze et al., 1975; Venyaminov & Kalnin, 1990). A comparison of the deconvolved spectra in Figure 2A,B reveals that the isotope-induced shift of the main amide I band contour from 1644 to 1589 cm^{-1} is accompanied by a narrowing of the overall band contour. Apart from the changes observed for the amide I band, the carboxyl groups of Glu and Asp in the protein also shifted about 43 cm^{-1} toward lower wavenumber as a result of ^{13}C substitution for ^{12}C (from 1580 cm^{-1} to 1536 cm^{-1} and from 1555 cm^{-1} to 1513 cm^{-1}).

Figure 3A–C shows the infrared spectra of $^{13}C/^{15}N$-uniformly labeled CaM and its complexes with the MLCK peptide, the cNOS peptide, and the CaD peptide, respectively. The data clearly show that the amide I bands from the three peptides are well separated from the amide I band of CaM. Hence, it is now possible to determine the secondary structure of the peptides and the protein separately in the complexes. The amide I bands at 1589 cm^{-1} of free CaM and the proteins complexed with the two target peptides are very similar, suggesting that the two globular domains of CaM retain almost the same secondary structure upon the formation of complexes with these peptides.

The conformational changes in the MLCK peptide, the cNOS peptide, and the CaD peptide upon binding to CaM were studied by comparing the infrared spectra of these three peptides in D_2O and in their complexes with $^{15}N/^{13}C$-uniformly labeled CaM. For further comparison, the infrared spectra of the three peptides in a 1/1 TFE/D_2O (v/v) mixture were

also acquired, since we have shown earlier by NMR that all three peptides can adopt a relatively stable α-helical conformation under these conditions, which resembles the structure of the CaM-bound peptide (Zhang et al., 1993; Zhang & Vogel, 1994a,b). Figure 4A shows the infrared spectra of the MLCK peptide in D_2O, the CaM/MLCK–peptide complex, and the peptide in 1/1 TFE/D_2O mixture. Since free CaM and CaM in the complex have very similar infrared spectra, the difference spectrum for the CaM/MLCK–peptide with free CaM is presented here to better represent the spectrum of the MLCK peptide in the complex. The carry-over counterion trifluoroacetic acid (TFA) used in the peptide synthesis, which has a strong and sharp band at 1674 cm^{-1}, was used as an internal reference to normalize the infrared spectra of the MLCK peptide obtained under the three different conditions. This permits one to compare not only frequencies but also the intensities between the spectra of the peptide in D_2O and in the corresponding peptide/CaM complexes. In D_2O solution, the amide I band of the MLCK peptide shows a relatively broad band centered around 1643 cm^{-1} which is typical for an unstructured peptide and is consistent with earlier studies by CD and NMR (Ikura & Bax, 1992; Zhang et al., 1993). The binding of this peptide to CaM induces significant changes in the position, as well as in the width, of the amide I band of the MLCK peptide. The amide I band of the MLCK peptide in the complex is considerably narrower, more intense, and centered at 1648 cm^{-1}, suggesting that the MLCK peptide changed from an irregular structure to an α-helical conformation upon binding to CaM. These changes could be reversed by addition of a calcium chelator (data not shown), demonstrating that the binding is calcium dependent. As expected, the amide I band of the MLCK peptide in 1/1 TFE/D_2O mixture is also centered at 1647 cm^{-1} and narrower than that in D_2O; this suggests that the MLCK peptide can adopt a similar α-helical structure in aqueous TFE solution as in the CaM complex (Zhang et al., 1993). Interestingly, certain spectral differences are also observed in the range 1560–1610 cm^{-1} (compare the solid and dashed lines in Figure 4A). The differences in this region observed between the spectrum of the free peptide in D_2O and the spectrum obtained by subtracting the spectrum of CaM from that of the CaM/MLCK peptide complex (solid line in Figure 4A) in all likelihood result from differences between the spectrum of free CaM and the spectrum of CaM in complex with the MLCK peptide. The data suggest that a slight but perceptible perturbation of the conformation of CaM occurs upon the binding of the peptide. Indeed, certain conformational changes in CaM, such as the unraveling and bending of the central helix of CaM upon formation of the complex, have been detected previously (Ikura et al., 1992; Meador et al., 1992, 1993). Such structural distortions may possibly account for the observed differences between the infrared spectra, although a reliable interpretation of the spectral changes in the amide I region of $^{13}C/^{15}N$-labeled CaM, or of other isotopically labeled proteins, requires further studies.

Figure 4B shows the infrared spectra of the cNOS peptide in D_2O, the CaM/cNOS–peptide complex, and the spectrum for the cNOS peptide in a TFE/D_2O mixture. Similar to the results obtained for the MLCK peptide, the cNOS peptide appears to have an irregular structure in D_2O solution as judged from the broad amide I band centered at 1643 cm^{-1}. Earlier CD and NMR studies have shown that the cNOS peptide adopts a predominantly irregular structure in aqueous solution (Zhang & Vogel, 1994a). The cNOS peptide binds to CaM in an α-helical conformation as its amide I band shifts to 1647 cm^{-1} and becomes narrower in bandwidth upon the formation
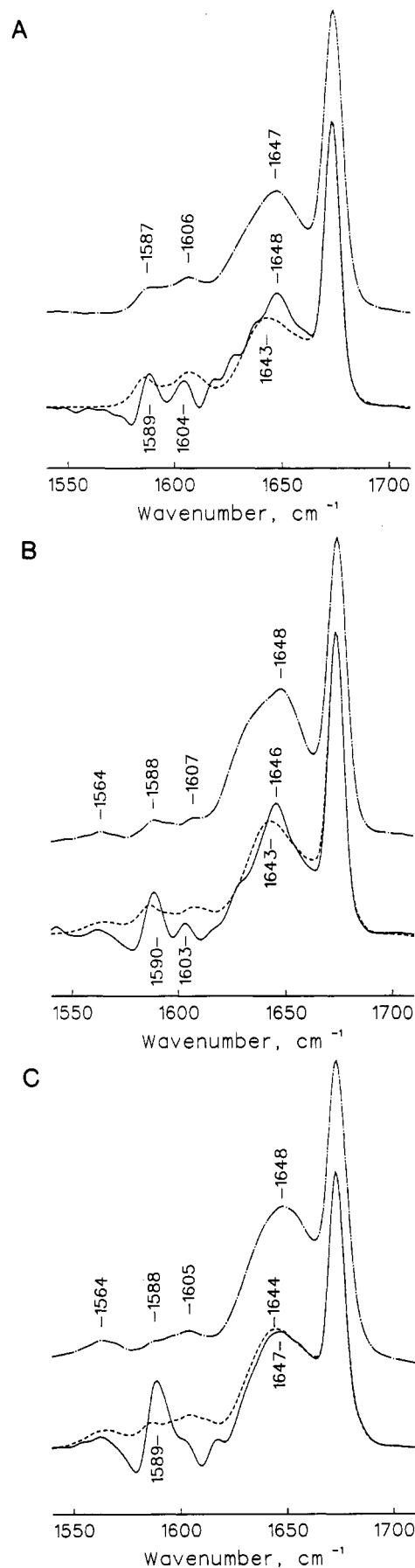

FIGURE 4: Deconvolved infrared spectra of the MLCK peptide (A) the cNOS peptide (B), and the CaD peptide (C) in D_2O (dashed lines), 1/1 TFE/D_2O mixture (dash-dotted lines), and the complexes with $^{15}N/^{13}C$-labeled CaM (solid lines), respectively. The infrared band resulting from internal trifluoroacetic acid was used to normalize the intensity of the corresponding peptide spectra.

of the complex with CaM. A similar α-helical structure can also be induced in the cNOS peptide by adding TFE into the aqueous peptide solution (see Figure 3B). These results are consistent with the earlier observation that the cNOS peptide binds to CaM in a manner very similar to that of the MLCK peptide (Zhang & Vogel, 1994a). Indeed, the spectral changes in the difference spectra of CaM that result from the binding of the cNOS peptide are also comparable to those observed for the CaM/MLCK peptide complex.

Figure 4C shows the interaction of the CaD peptide with the uniformly labeled CaM. The infrared spectra of the CaD peptide show similar overall changes upon complex formation with CaM or the addition of TFE, compared to what is observed for the MLCK peptide and the cNOS peptide. However, the spectral changes of the CaD peptide are less dramatic, reflecting the fact that the CaD peptide binds to CaM with a much lower affinity ($\approx 10^3$-fold lower) and that the α-helix of the CaD peptide in the CaM/CaD–peptide complex is much shorter (≈ 10 residues) (Zhang & Vogel, 1994b). On the other hand, clear changes in the range 1570–1610 cm^{-1} of the difference spectrum obtained by subtracting the absorbance spectrum of CaM from that of the CaM/CaD peptide complex (solid line in Figure 4C) suggest a somewhat more significant perturbation of the secondary structure of CaM upon the binding of the CaD peptide than those induced by the binding of the MLCK peptide and the cNOS peptide.

DISCUSSION

FTIR spectroscopy has shown considerable potential for determining the secondary structure in proteins and peptides (Surewicz & Mantsch, 1988; Surewicz et al., 1993). In principle, this technique should also be well suited for investigating conformational changes that occur during protein–protein or protein–peptide interactions. However, extensive overlap of the diagnostic infrared bands limits the application of FTIR spectroscopy for the study of conformational changes of each component when two proteins (peptides) form a complex. In a few studies, specific isotope-labeling of proteins and peptides has been used to increase the resolution in infrared spectra. For example, Tadesse et al. (1991) have shown that specific ^{13}C labeling of the Ala and Gly carbonyl carbons in a peptide resulted in a shift of ≈ 36 cm^{-1} for their respective amide I bands. This isotope-labeling strategy enabled these authors to examine the secondary structure of amino acid residues at specific locations in this conformationally heterogeneous peptide. Furthermore, carbon-13, nitrogen-15, and deuterium labeling of specific groups in the side chains of certain amino acid residues has also been used to study the role of these residues in different states of bacteriorhodopsin (Roepe et al., 1987a,b; Rothschild et al., 1989, and references therein). More recently, it was shown that extensive labeling of a protein with ^{13}C leads to a significant downshift of the protein's amide I band (Harris et al., 1992).

Fourier transform infrared spectroscopy has been used earlier to study the secondary structure and metal ion induced conformational changes of CaM (Trewhella et al., 1989; Jackson et al., 1991). In this work, we have used uniformly ^{13}C-labeled CaM to study the interaction of the protein with target peptides by infrared spectroscopy. These target peptides have the same amino acid sequence as the CaM-binding domains from various proteins. The data presented in Figures 3 and 4 clearly show that uniform ^{13}C labeling of CaM allows one to individually study the conformational changes of the target peptides and the protein in the complex, since their amide I bands are well separated. It is noteworthy that we

obtained a shift of 55 cm^{-1} for the amide I band, which is at least 10 cm^{-1} more than was observed for other proteins (Harris et al., 1992); the rationale for this difference is unclear at present. We conclude that CaM largely retains its secondary structure upon complex formation; however, some subtle changes could be detected (Figure 4). These results are in keeping with the earlier observed structures of CaM in complexes with its target peptides (Roth et al., 1991; Ikura et al., 1992; Meador et al., 1992, 1993). Moreover, our results show that the three target peptides studied here all bind in an α-helical conformation to CaM. Unlike CD spectroscopy where bands for the protein and the peptide cannot be separated, the "isotope-edited" FTIR approach has allowed us to determine the secondary structure of the CaM-bound MLCK peptide, cNOS peptide, and CaD peptide without ambiguity; the results obtained here are in excellent agreement with those derived earlier using other more involved experimental approaches (Meador et al., 1992; Ikura & Bax, 1992; Zhang et al., 1993; Zhang & Vogel, 1994a,b). Thus, in principle it should be possible now to determine the secondary structure of target peptides from other CaM–target proteins, of which the CaM-bound structures are not available, in the same fashion. That such studies are necessary is indicated by the recent observation that peptides with high β-sheet forming potential were found to bind tightly to CaM (Dedman et al., 1993), illustrating that target peptides may have different secondary structures when bound to CaM. Using the same approach, it might be possible to study complexes of CaM with intact target proteins; such studies could help define whether the CaM-binding domains have a different secondary structure when bound to CaM or as part of the target protein, as appears to be the case for MLCK (Zhang et al., 1993). Due to the advances in the area of recombinant DNA technology and the numerous possibilities for efficient biosynthetic isotopic labeling of proteins, the isotope-shifting strategy presented in this work should also be generally applicable to study a wide variety of other protein–protein and protein–peptide interactions. In addition, since the conformation-sensitive carbonyl vibrations of nucleic acids (Taillandier & Liquier, 1992) also overlap with the amide I band of the protein, the same "isotope-editing" approach should prove useful for studying protein-nucleic acid interactions.

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