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Comparative analysis of the amino- and carboxy-terminal domains of calmodulin by Fourier transform infrared spectroscopy

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Abstract. Fourier transform infrared spectra were obtained for mammalian calmodulin and two of its fragments produced by limited proteolysis with trypsin TR_1C (1–77) and TR_2C (78–148). Experiments were done in H_2O, D_2O and D_2O/trifluoroethanol (TFE) mixtures. Information about secondary structure was obtained from analysis of the amide I and II bands; while characteristic absorbances for tyrosine, phenylalanine and carboxylate groups were analyzed for changes in tertiary structure. Our data indicate that the secondary and tertiary structure is preserved in the two half molecules of CaM, both in the apo- and Ca^{2+}-saturated state. Addition of the structure-inducing solvent TFE causes marked changes only in the apo-TR_1C domain. The maximum wavenumber for the amide I band of the two domains of CaM in D_2O was markedly different (1642 cm^{-1} for TR_1C versus 1646/1648 cm^{-1} for Ca^{2+} and apo-TR_2C). This renders the amide I band for the intact protein very broad in comparison to that in other proteins and is indicative of a distribution of α-helices with slightly different hydrogen bonding patterns.

Key words: Calmodulin – Calmodulin fragments – FTIR spectroscopy – Ca^{2+}-binding effects

1. Introduction

Calmodulin (CaM) is a small calcium-binding protein that functions as a calcium signal transducer in eukaryotic cells. It is a versatile protein that can activate at least 30 different target systems (Vogel 1994). X-ray studies (Babu et al. 1988) have shown that the calcium saturated protein has two globular domains which are connected by a long, solvent-exposed α-helix. This long helical tether is quite flexible in solution as revealed by NMR spectroscopy (Ikura et al. 1991; Barbato et al. 1992). The characteristic two domain structure is also found in apo CaM (Zhang et al. 1995; Kuboniwa et al. 1995). Each of the two globular domains fully retains its Ca^{2+}-binding activity and ability to bind small hydrophobic molecules (Vogel et al. 1983; Andersson et al. 1983; Linse et al. 1991). Thus it is not unrealistic to expect that the biophysical properties of the two domains resemble those of intact CaM.

In order to address the question to what extent the two domains of CaM resemble the intact protein, we have undertaken an FTIR spectroscopic study of both domains of CaM. FTIR spectroscopy has emerged as a powerful technique to characterize conformational differences between related proteins by analyzing the backbone conformation-sensitive amide I bands and the infrared absorption bands of certain amino acid side-chains (Perrier et al. 1994; Fabian et al. 1994). In combination with isotope labeling, the technique also permits the study of protein-peptide interactions (Zhang et al. 1994). Herein, we have measured the infrared spectra of the amino-terminal domain (TR_1C) and of the carboxy-terminal domain (TR_2C) of mammalian CaM overexpressed in *E. coli*, under various solvent conditions (in H_2O- or D_2O-buffer and in the presence of the structure-inducing solvent trifluoroethanol). The spectra of the two fragments were compared with the spectrum of intact CaM obtained under strictly comparative experimental conditions. Our results demonstrate that the infrared spectra of the two domains of CaM are perfectly additive.

2. Materials and methods

CaM was purified from an *E. coli* strain harbouring a synthetic mammalian CaM-gene, using a published expression and purification protocol (Waltersson et al. 1993; Zhang and Vogel 1993). Proteolytic fragments of CaM were prepared by limited digestion of the calcium protein with trypsin (Andersson et al. 1983; Vogel et al. 1983). This procedure cuts calmodulin in the central linker region and generally produces a mixture of the C-terminal domain TR_2C (78–148, 76–148, 75–148) and a mixture of N-terminal domain fragments TR_1C (1–77, 1–75, 1–74)

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(Thulin et al. 1984); the major products are TR_1C (1–77) and TR_2C (78–148). The proteolytic fragments were separated from remaining CaM and the trypsin/soybean-trypsin-inhibitor complex by chromatography on Sephadex G-50 (50 mM NH_3HCO_3, 50 mM NaCl, pH 7.9). The fragments are purified by hydrophobic interaction chromatography on phenyl-Sepharose. Both half-molecules bind to phenyl-Sepharose in 50 mM TRIS, 1 mM $CaCl_2$, pH 7.5. The TR_2C mixture elutes in 2 mM TRIS, 1 mM $CaCl_2$, pH 7.5 and the TR_1C mixture elutes with distilled water, pH 7.5. Proton NMR spectra of the fragments (data not shown) used for the FTIR studies were identical to those reported earlier (Thulin et al. 1984; Ikura et al. 1984). The apo-form of calmodulin and the two apo-fragments were prepared by passage through a Chelex-100 column. Proton NMR was used to confirm that the protein was in its apo-form. FTIR measurements of apo protein samples were completed within 20 min in order to eliminate the possibility of significant Ca^{2+}-leaking from the infrared cell.

Infrared spectroscopy

The protein solutions were placed between a pair of CaF_2 windows separated by 6 μM for measurements in H_2O-buffer (50 mM HEPES, pH 7.5), or by 45 μM for samples in D_2O-buffer. For FTIR measurements in D_2O, the apo-form of CaM was left overnight at room temperature to ensure the complete exchange of all amide proteins with deuterons. The Ca^{2+}-form of CaM was then prepared by adding the appropriate amount of $CaCl_2$ to the apo-CaM solution as described by Zhang et al. (1994). Infrared spectra were recorded on a Digilab FTS-40A FTIR spectrometer equipped with a liquid nitrogen cooled mercury cadmium telluride detector and continuously purged with dry air. For each sample, 512 interferograms were co-added and Fourier-transformed to generate a spectrum with a nominal resolution of 2 cm^{-1}. Pure solvent spectra were recorded under identical conditions and subtracted from the spectra of the proteins in the relevant solvent. Spectral contributions from residual water vapour were eliminated using a set of water vapour spectra measured under identical conditions. The subtraction factor was varied until the second derivative of the absorption in the range 1750–1850 cm^{-1} was featureless, thereby avoiding artificial bands and/or incorrect band positions in the amide I and amide II region of the protein spectrum (Jackson and Mantsch 1995). The final unsmoothed protein spectra were used for further analysis. Band narrowing by Fourier self-deconvolution was performed as described previously (Mantsch et al. 1988) using a half bandwidth of 16 cm^{-1} and a band-narrowing factor k = 2.0. Amplitudes of all spectra were normalised to the maximum of the amide I absorption to facilitate comparison.

3. Results

Figure 1 shows the infrared spectra of intact CaM, a 1:1 mixture of TR_1C and TR_2C, and the two domains alone,

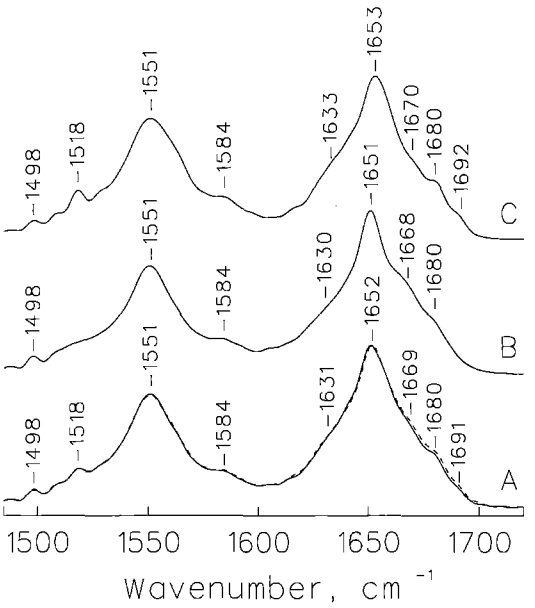

Fig. 1. A Comparison of the infrared spectrum in the amide I and amide II region of intact CaM (solid line) with the spectrum of a 1:1 mixture of TR_1C and TR_2C (dashed line) both recorded in H_2O-buffer saturated with Ca^{2+}; **B** infrared spectrum of TR_1C, and **C** infrared spectrum of TR_2C, measured under the same experimental conditions. All spectra are shown after Fourier self-deconvolution performed with the parameters given under Materials and Methods

each in their Ca^{2+}-saturated state in H_2O. The major absorption bands are the amide I band centered at ~1652 cm^{-1} and the amide II band centered at 1551 cm^{-1}. The dominant amide I band component at 1652 cm^{-1} in the spectrum of intact CaM reflects the known high content of α-helical structure (~65%). The weaker bands at 1669 and 1680 cm^{-1} are assigned to "turn-like" structures (Jackson and Mantsch 1995) known to be present in CaM (Babu et al. 1988). A small amount of β-structure is indicated by the shoulder band at ~1630 cm^{-1}. At most 12 residues, or 8% of intact CaM, are known to be present in two antiparallel mini β-sheets formed between the two Ca^{2+}-binding loops in each domain of the protein (Babu et al. 1988; Ikura et al. 1991). In the case of CaM, such a small amount of β-structure in an otherwise predominantly α-helical protein is hard to detect by infrared spectroscopy, even after band-narrowing by Fourier self-deconvolution. The bands at 1584, 1518, and 1498 cm^{-1} are due to amino acid side-chain absorptions: phenylalanine (1498 cm^{-1}), tyrosine (1518 cm^{-1}), and aspartate (1584 cm^{-1}) (Venyaminov and Kalnin 1990). A comparison with the infrared spectra in Figs. 1 B and 1 C reveals that the spectrum of the amino-terminal domain lacks the tyrosine band. This agrees with the fact that the TR_1C fragment contains five phenylalanine residues and no tyrosine, while the TR_2C fragment has three phenylalanine and two tyrosine residues (Klee and Vanaman 1982). Careful examination of the spectra in Fig. 1 reveals other minor differences between the spectra of the two domains in the conformation-sensitive amide I region. The major amide I band component is located at 1651 cm^{-1} in the spectrum of TR_1C and at 1653 cm^{-1} in the spectrum of TR_2C. In addition, a shoulder at 1692 cm^{-1}

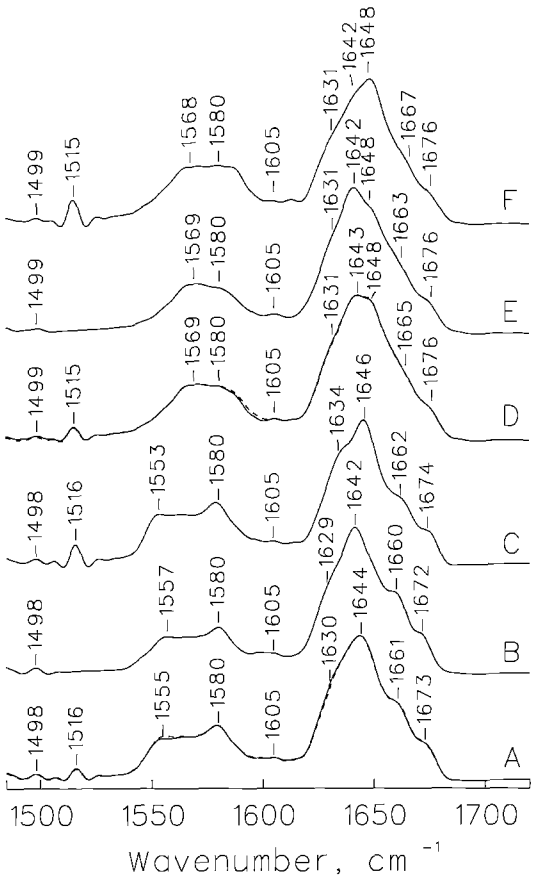

Fig. 2. **A** Infrared spectra of intact CaM (solid line) and of a 1:1 mixture of TR_1C and TR_2C (dashed line) in D_2O-buffer saturated with Ca^{2+}; **B** spectrum of TR_1C and **C** spectrum of TR_2C, both measured under the same experimental conditions. **D** Infrared spectra of apo-CaM (solid line) and a 1:1 mixture of apo-TR_1C and apo-TR_2C (dashed line) in D_2O-buffer; **E** spectrum of apo-TR_1C and **F** spectrum of apo-TR_2C, both measured under the same experimental conditions. All spectra are shown after Fourier self-deconvolution

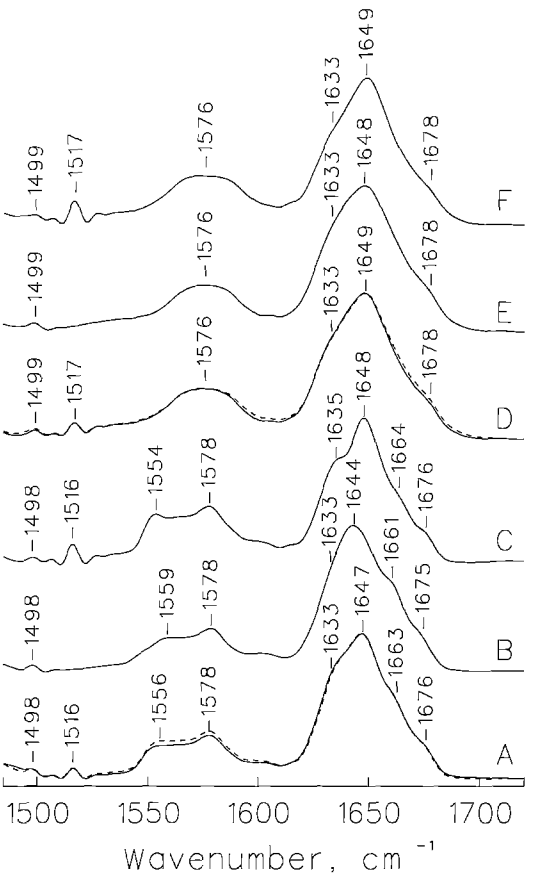

Fig. 3. **A** Infrared spectra (after Fourier self-deconvolution) of intact CaM (solid line) and of a 1:1 mixture of TR_1C and TR_2C (dashed line) in 1:1 TFE/D_2O saturated with Ca^{2+}; **B** spectrum of TR_1C, and **C** spectrum of TR_2C, both measured under the same experimental conditions. **D** Infrared spectra (after Fourier self-deconvolution) of intact apo-CaM (solid line) and a 1:1 mixture of apo-TR_1C and apo-TR_2C (dashed line) in 1:1 TFE/D_2O; **E** spectrum of apo-TR_1C, and **F** spectrum of apo-TR_2C, both measured under the same experimental conditions

is observed only in the spectrum of the carboxy-terminal domain (see Fig. 1 C). Interestingly, the infrared spectrum of the 1:1 mixture of the two domains is practically identical with the spectrum of intact CaM (compare the solid and dashed lines in Fig. 1).

To expand the comparative analysis, we also measured infrared spectra of the fragments TR_1C and TR_2C, and of other 1:1 mixtures in D_2O and in 50% TFE/D_2O. Spectra of proteins in D_2O solutions have the advantage of permitting a clear separation of side-chain absorptions from the protein backbone amide II bands (both of which occur in the 1500–1600 cm^{-1} region) as the latter are shifted to 1450 cm^{-1} in ^1HN-^2HN exchanged proteins. Therefore, all other measurements were carried out with D_2O-buffered solutions. Infrared bands that remain in the range 1550–1580 cm^{-1} after complete ^1HN-^2HN exchange arise from the antisymmetric COO^- stretching vibrations of the carboxylate moiety of the amino acid side-chain groups of glutamate and aspartate. In calmodulin, 14 out of the 38 COO^- groups are found in the Ca^{2+}-binding sites, and the spectral features shown in Fig. 2 A are highly characteristic for the Ca^{2+}-bound form of CaM (Nara et al. 1995).

The minor spectral differences near 1555 cm^{-1} between the infrared spectrum of intact CaM and that of the 1:1 mixture of the two domains, may reflect only slight, if any differences in the microenvironment of a few COO^- groups. Furthermore, a comparison of the spectra in the range 1490–1520 cm^{-1} indicates that there are also no detectable differences between the microenvironment of the aromatic amino acids in intact CaM in comparison to those in the 1:1 mixture of TR_1C and TR_2C. Infrared bands in this region, in particular the frequency of the aromatic ring stretching vibration of tyrosine at ~1516 cm^{-1}, are sensitive local monitors of the protein conformation (Fabian et al. 1994). The spectrum in the amide I region of intact CaM and that of the mixture of the two domains also are practically identical (compare the solid and dashed line in Fig. 2 A). Furthermore, a synthetic spectrum of CaM, generated by summation (with appropriate weighting) of the spectra of TR_1C and TR_2C is also identical with the spectra in Fig. 2 A. The peak positions of the amide I band components for the different forms of CaM are summarized in Table 1.

Table 1. Peak positions of the amide I band components for the different forms of CaM in D_2O-buffer and in 1:1 TFE/D_2O, respectively

Component positions (cm^{-1}) in D_2O-buffer				
intact CaM, Ca^{2+}	1630	1644	1661	1673
1:1 mixture, Ca^{2+}	1630	1644	1661	1673
TR_1C, Ca^{2+}	1629	1642	1660	1672
TR_2C, Ca^{2+}	1634	1646	1662	1674
intact Apo-CaM	1631	1643/1648	1665	1676
1:1 mixture	1631	1643/1648	1665	1676
Apo-TR_1C	1631	1642 (1648)	1663	1676
Apo-TR_2C	1633	(1642) 1648	1667	1676
Component positions (cm^{-1}) in 1:1 TFE/D_2O				
intact CaM, Ca^{2+}	1633	1647	1663	1676
1:1 mixture, Ca^{2+}	1633	1647	1663	1676
TR_1C, Ca^{2+}		1644	1661	1675
TR_2C, Ca^{2+}	1635	1648	1664	1676
intact Apo-CaM	1633	1649		1678
1:1 mixture	1633	1649		1678
Apo-TR_1C	1633	1648		1678
Apo-TR_2C	1633	1649		1678

Assignments: The infrared bands at 1629–1635 cm^{-1} can be assigned to β-sheet structures. Turn structures are associated with the band components in the range 1660–1678 cm^{-1}. The dominant band component(s) at 1642–1649 cm^{-1} reflect(s) the presence of α-helical structures in CaM. Minor contributions from irregular structures, 3_{10}-helical and β-turn structures all present in CaM can also be expected in the range 1640–1650 cm^{-1}.

While the infrared spectra of the two domains of CaM are perfectly additive, the spectra of the domains themselves reveal some interesting spectral differences. Thus, the major amide I is located at 1642 cm^{-1} in the spectrum of the amino-terminal domain, but at 1646 cm^{-1} in the spectrum of the carboxy-terminal domain. A comparison of the spectra of the two domains in the presence of the structure-inducing solvent trifluoroethanol reveals a 2 cm^{-1} shift of the amide I band to higher wavenumbers. Furthermore, the infrared spectrum of the 1:1 mixture of TR_1C and TR_2C is practically identical to the spectrum of intact CaM (compare the solid and dashed lines in Fig. 3 A).

We also compared the infrared spectra for the apo-protein and the corresponding two half-molecules. Again, the infrared spectrum of the 1:1 mixture of apo-TR_1C and apo-TR_2C perfectly matches that of intact apo-CaM, both in D_2O (Fig. 2 D) and in 1:1 TFE/D_2O (Fig. 3 D). The infrared spectra of the two individual domains reveal clear differences among themselves as well as in comparison with the spectrum of the Ca^{2+}-saturated form recorded under identical conditions. In D_2O-buffer, the major amide I band is located at 1642 cm^{-1} in the spectrum of the amino-terminal domain (Fig. 2 E), which coincides with the position found in the Ca^{2+}-saturated form (Fig. 2 B). On the other hand, in the spectrum of the carboxy-terminal domain (Fig. 2 F), this band is at 1648 cm^{-1} and thus 2 cm^{-1} higher than in the case of the Ca^{2+}-saturated TR_2C fragment (Fig. 2 C). This indicates that the conformational changes induced by Ca^{2+} are greater in TR_2C than in TR_1C, in agreement with CD studies by Martin and Bayley (1986). The different position of the maximum of the amide I bands of the two domains results in an apparently broad amide I band contour of intact apo-CaM (compare traces in Fig. 2 D with those in Fig. 2 E and F). When comparing the amide I bands of the apo-form (Fig. 2 D) with those of the Ca^{2+}-saturated form of intact CaM (Fig. 2 A), the broader amide I band contour of the apo-form could easily be interpreted as simply an increase of unordered peptide moieties in the apo structure, at the expense of some α-helical peptide moieties in the Ca^{2+}-containing protein. On the basis of the present FTIR data obtained from the two domains, however, this broader amide I band of the apo-form of intact CaM is more likely the result of slight conformational differences that exist between the apo-form and the Ca^{2+}-form of the TR_2C fragment, though an increase in α-helicity in intact CaM in the presence of Ca^{2+} cannot be ruled on the basis of the infrared spectra alone.

Further differences between the infrared spectra of the apo- and the Ca^{2+}-forms of CaM are observed in the range 1490–1600 cm^{-1}. Thus, the antisymmetric COO^- stretching modes are highly characteristic for the Ca^{2+}-free form of the COO^--groups in CaM (Nara et al. 1995). In particular, the 1555 cm^{-1} band of the Ca^{2+}-form of CaM (Fig. 2 A) reflects the coordination of the COO^- groups of Glu residues to Ca^{2+} ions (Nara et al. 1994); the corresponding band of "free" glutamate is located at 1569 cm^{-1} (Fig. 2 D). Small differences exist in the microenvironment of some tyrosine and phenylalanine residues as indicated by a comparison of the corresponding infrared marker bands. This effect was also detected by near UV-CD and NMR (Martin and Bayley 1986; Thulin et al. 1984). The infrared tyrosine marker band is located at 1515 cm^{-1} in the spectrum of the apo-form, but at 1516 cm^{-1} in the spectrum of the Ca^{2+}-form. Since the two tyrosines in CaM are located in the carboxy-terminal domain, this indicates conformational changes induced by binding of Ca^{2+} in this domain, in good agreement with the conclusions derived from the comparison of the spectra in the amide I region. TR_1C contains five phenylalanine residues while the TR_2C fragment has only three. The slightly different position of the phenylalanine marker band (1498 cm^{-1} in the Ca^{2+}-form form versus 1499 cm^{-1} in the Ca^{2+}-free form) suggests some differences in the microenvironment of phenylalanine residues in TR_1C alone or in both domains due to Ca^{2+}-binding.

While the addition of TFE to the isolated domains and to the intact protein has no major impact on the Ca^{2+}-saturated forms, it clearly affects the conformation of the apo-form of the amino-terminal domain. A comparison of the spectra of apo-TR_1C reveals a 6 cm^{-1} shift in the amide I frequency in the presence of TFE (compare Figs. 2 E and 3 E). On the other hand, the spectra of apo-TR_2C are almost identical in the absence or presence of TFE (compare Figs. 2 F and 3 F). Furthermore, the addition of the structure-inducing solvent TFE leads to almost identical spectra for the apo-forms of the two domains and of intact apo-CaM (Fig. 3 D). This suggests that the conformation of both the half-molecules and the intact protein is stabilized in the presence of Ca^{2+}, while significant conformational

changes can be induced in the apo-form for the TR_1C fragment by addition of trifluoroethanol.

4. Discussion

Proteolytic fragments of calmodulin have been produced by numerous investigators. In particular they have been used to determine the parts of calmodulin that are crucial for activating a range of different target enzymes (Walsh et al. 1977; Kuznicki et al. 1981; Newton et al. 1984; Minowa et al. 1988). In most instances it was found that isolated half-molecule fragments were unable to substitute for intact CaM; although they did not activate they could still bind to the target proteins (Ni and Klee 1985). More recently, enzyme activation studies have utilized combinations of TR_1C and TR_2C which gave rise to substantial activation compared to intact CaM (Persechini et al. 1994). These studies indicate that both domains of CaM are generally required for enzyme activation, a feature which is totally consistent with the solution and crystal structures determined for CaM complexed to target peptides (Ikura et al. 1992; Meador et al. 1992, 1993) where the two domains of CaM bind to the target peptide and stabilize its α-helical structure through amino acid side-chain interactions.

Various studies have been reported in which the properties of the two half-molecules have been compared to intact CaM. Both fragments retain the capacity of CaM to expose a hydrophobic surface upon Ca^{2+} binding (Vogel et al. 1983), they bind metal ions normally (Andersson et al. 1983; Martin and Bayley 1986; Linse et al. 1991) and like intact CaM they are markedly stabilized against denaturation by calcium binding (Brzeska et al. 1983; Tsalkova and Privalov 1985; Martin and Bayley 1986). Moreover, in the calcium form they largely retain their secondary and tertiary structure, as revealed by circular dichroism, fluorescence, proton NMR and X-ray crystallographic studies (Drabikowski et al. 1982; Thulin et al. 1984; Ikura et al. 1984; Martin and Bayley 1986; Sjölin et al. 1990). Subtle differences may exist between the structures of the apo-fragments and intact apo-calmodulin (Drabikowski et al. 1982; Thulin et al. 1984), nevertheless, the solution structure for the isolated apo-TR_2C fragment of CaM has been determined (Finn et al. 1993), in an attempt to understand the conformational transitions upon calcium binding in CaM.

Our FTIR results for the Ca^{2+}-saturated forms of CaM, TR_1C and TR_2C are in excellent agreement with the earlier studies. The spectra for the two domains add up to match closely that of the intact protein. From this we conclude that the secondary structure of the individual domains is preserved in the intact calmodulin molecule. We note with interest that the addition of TFE to the isolated Ca^{2+}-saturated domains and the intact protein does not cause a large increase in the secondary structure for the Ca^{2+}-saturated forms CaM. Thus there is no drastic change in the protein structure in this environment, unlike what is seen for several other proteins (see for example Fan et al. 1993; Buck et al. 1993). This is in agreement with recent CD studies, where a small increase in α-helicity was observed upon addition of TFE. This was tentatively assigned to a stabilization of α-helical structure in the central linker region of CaM (Bayley and Martin1992). Such a change from random extended structure to α-helical structure in a small portion of a protein is difficult to detect by FTIR, since the wavenumbers for the two structural elements are relatively close (Jackson and Mantsch 1995). However, since TFE appears to have almost no effect on the secondary structure of the two isolated Ca^{2+}-saturated domains (see Fig. 3), our data are consistent with this interpretation of the CD data. In fact, the 2 cm^{-1} change of the amide I band in TR_2C (78–148) upon addition of TFE could be related to stabilization of α-helix in the region 78–82, which is the unstructured part of the linker in intact CaM (Ikura et al. 1991; Barbato et al. 1992).

We have also compared the FTIR spectra for the apoprotein and the two half molecules; again we note that there is excellent agreement between the fragments and the intact protein. This suggests preservation of secondary structure in the half-molecules; thus validating their use in total structure determination by NMR (Finn et al. 1993).

Finally we note that the maxima of the amide I bands of the TR_1C and TR_2C fragments are markedly different. In fact, for TR_1C the maximum is slightly outside of the range which is normally considered to be diagnostic for α-helical structures. Though amide I bands between 1650 and 1658 cm^{-1} are generally considered to be characteristic of α-helical structures (Byler and Susi 1986), there exist a number of proteins for which the standard correlations of amide I band position and structure do not appear to hold. This feature is common not only to calmodulin but also to adenylate kinase, troponin C, and parvalbumin. The position of the amide I maximum of this latter group of proteins known to be highly α-helical, occurring at 1652–1645 cm^{-1} (Jackson and Mantsch 1995) is actually more consistent with a highly unordered secondary structure. The helical structure of poly-L-lysine even exhibits an amide I band at ~1630 cm^{-1} (Jackson et al. 1989). Possible interpretations of atypical amide I band positions, such as an unusual degree of solvent-protein interaction or distortion of structural elements were discussed (Trewhella et al. 1989; Jackson and Mantsch 1995). In the case of TR_1C and TR_2C, the available X-ray and NMR data for the intact calcium-saturated protein demonstrate the presence of similar α-helical structures in these two domains, while the slightly different positions of their amide I infrared bands suggest distinct differences in the hydrogen-bonding pattern of the amide C=O groups in the four α-helices of TR_1C in comparison to the overall pattern of the corresponding groups in TR_2C. Also in the recently determined solution structure of apo-CaM (Finn et al. 1995; Kubinowa et al. 1995; Zhang et al. 1995; M. Ikura, personal communication) no significant differences were found in the NMR parameters of α helices in the N- and C-terminal domain. One example of a structural difference is the presence of a pronounced kink in helix B of the N-terminal domain, but its absence in the homologous helix F of the C-terminal domain. Furthermore, the backbone amide protons in the C-terminal domain of apo-CaM were found to be considerably less stable to hydrogen exchange than in the N-terminal domain (Kubinowa et al. 1995). While

not diagnostic by themselves, the differences observed between the infrared spectra of apo-TR_1C and apo-TR_2C would be compatible with such conformational differences. The different maximum amide I positions for the two domains also contribute to the overall very broad and therefore uncharacteristic band shape for this predominantly α-helical protein. This broad and less structured band shape in the amide I region does not allow a reliable quantitation of the relative amounts of particular structural elements in the different forms of CaM using curve-fitting procedures, even when extensive resolution enhancement is applied.

Furthermore, the difficulties of obtaining absolute intensity standards for the FTIR spectra of native and denatured calmodulin also restrict quantitative comparison of the secondary structural changes induced by calcium binding by infrared difference spectroscopy. In some of our recent papers, the FTIR spectra of thermally unfolded proteins have been used as intensity standard for the quantitative comparison of the spectra of the corresponding folded proteins (Fabian et al. 1994; Perrier et al. 1994). The great thermal stability of the Ca^{2+}-forms of CaM prevents such experiments in the present case.

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