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Protein engineering and NMR studies of calmodulin

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Abstract

The calcium regulatory protein calmodulin (CaM) plays a role as an on-off switch in the activation of many enzymes and proteins. CaM has a dumbbell shaped structure with two folded domains, which are connected by a flexible linker in solution. The calmodulin-binding domains of the target proteins are contained in 20 residue long amino acid sequences, that share no obvious amino acid sequence homology. In this contribution, we discuss the features of CaM, which allow it to be rather promiscuous, and bind effectively to all these distinct domains. In particular, we describe the role of the methionine-rich hydrophobic surfaces of the protein in providing a malleable and sticky surface for binding many hydrophobic peptides. The enzyme activation properties of various Met → Leu mutants of CaM are discussed. In addition, the role of the flexible linker region that connects the two domains is also analyzed. Finally, we describe various NMR and spectroscopic experiments that aid in determining the CaM-bound structures of synthetic peptides containing various CaM-binding domains. All structures analyzed to date are α-helical when bound to CaM, and they interact with CaM only through amino acid sidechains. This form of protein-protein interaction is rather unique, and may contribute to CaM's capacity to bind effectively to such a wide range of distinct partners. (Mol Cell Biochem **149/150:** 3–15, 1995)

Key words: calmodulin, calcium NMR studies, methionine protein-protein interaction

Abbreviations: CaM – calmodulin; CD – circular dichroism; cNOS – constitutive Nitric Oxide Synthase; FTIR – Fourier Transform Infrared Spectroscopy; iNOS – inducible Nitric Oxide Synthase; MLCK – Myosin Light Chain Kinase; NMR – Nuclear Magnetic Resonance; nOe – nuclear Overhauser effect; NOESY – two dimensional Nuclear Overhauser Effect Spectroscopy; PDE – cyclic nucleotide phosphodiesterase; SeMet, selenomethionine; TFE – trifluoroethanol; TOCSY – two dimensional Total Correlation Spectroscopy; trnOe – transferred nuclear Overhauser effect

Introduction

The regulation of many activities of a cell is controlled by various signal transduction pathways. These generally involve intracellular events such as protein phosphorylation, to switch specific proteins and enzymes on or off. Likewise, the intercellular messenger nitric oxide can readily diffuse to neighbouring cells and pass on regulatory information from one cell to others. Out of necessity, the activity of such pathways has to be strictly controlled. In the case of protein phosphorylation, the activity of the protein kinases and protein phosphatases, which phosphorylate and dephosphorylate the regulated proteins respectively at specific sites, is controlled by increasing levels of cyclic nucleotides or by calcium. In turn, the levels of cyclic nucleotides are also controlled through the influence of calcium on their biosynthetic and degrading enzymes. Nitric oxide synthases are also stimulated by an influx of calcium in the cell. These examples all indicate the central role that calcium plays in the control of signal transduction.

Calcium is also a well known regulator of muscle contraction. In skeletal and cardiac tissues this regulation is mediated through troponin C. This protein forms part of the thin filaments, and it is thought to undergo a large conformational change upon binding calcium. The skeletal muscle TnC protein possesses four calcium binding sites, and an increase in the cellular calcium concentration will give rise to activation of the muscle contraction. The four calcium ions are each

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bound in characteristic and homologous helix-loop-helix motifs [1]. A similar cardiac TnC protein, which only possesses three calcium-binding sites regulates the contractility of cardiac muscle. In contrast, the calcium activated contraction of smooth muscle tissue, is mediated by calmodulin, a calcium binding protein that is not continuously bound to the other components of the contractile machinery [1]. In fact, it will only bind to its target protein myosin light chain kinase (MLCK), after it has bound calcium. However, unlike the TnC's, CaM is not simply a specific activator of smooth muscle. In contrast, while the TnC proteins each only have one specific target, apart from activating smooth muscle MLCK, CaM has a wide range of other target enzymes and proteins [2]. In fact, the calcium calmodulin complex is responsible for the activation of many enzymes in cyclic nucleotide metabolism, several protein kinases and the nitric oxide synthases discussed in the previous paragraph [3]. It is also involved in learning processes in the brain [4, 5]. In addition, CaM is capable of activating some 25 other systems (Table 1, for recent review see [3]). This immediately raises the question as to how a single relatively small protein, such as calmodulin, can activate so many different target systems? To answer this question, it might be instructive to consider the situation for the protein kinases, most of which also have multiple targets. These enzymes all recognize specific 6–8 residue long amino acid sequences in their target proteins. For example, the cyclic AMP dependent protein kinase, will only phosphorylate the hydroxyl groups of Ser or Thr residues that are present in the sequence RRXSY, where X can be any residue and Y is a bulky aliphatic hydrophobic residue. Other protein kinases recognize other specific amino acid sequences [6]. Thus the high specificity of these reactions is provided by the high sequence homologies in the regulatory phosphorylation sites. All these protein kinases contain a complementary surface, comprising appropriately placed charged and hydrophobic residues around their catalyte site [7], which is designed for the specific binding of the recognition sequences. By analogy, one would expect that all CaM binding proteins would contain a homologous recognition amino acid sequence. This has however not been found. Although most CaM-binding domains are found in ≈ 20 amino acid residue long regions of the target proteins, there is almost no amino acid sequence homology between them (see Table 2). The only features shared by the CaM-binding domains, is that they are basic in nature, that acidic residues are virtually absent, and that they have a predicted tendency to form amphiphilic α-helices [8].

Table I. Some CaM-regulated enzymes and proteins grouped by their functions

Cell messengers
- plasma membrane Ca^{2+}-ATPase
- cyclic nucleotidephosphodiesterase
- adenylate cyclase
- nitric oxide synthase
- phospholamban (regulates SR-Ca^{2+}-ATPase)

Protein phosphorylation and dephosphorylation
- CaM kinase
- phosphorylase kinase
- calcineurin

Muscle contraction
- myosin light chain kinase (smooth and skeletal muscle)
- caldesmon

Gene expression and cell proliferation
- CaM kinase II
- CaM Binding proteins in nucleus
- CaM-dependent endonucleases
- Hsp90 (heat shock protein)

Others
- phosphofructokinase
- IP_3 kinase
- NAD kinase
- Hsp70
- B50 (neuromodulin)
- HIV transmembrane glycoprotein

Structures of calmodulin

In order to understand the function of CaM, we should first take a look at its structure. Early NMR experiments with proteolytic fragments of CaM, had provided some hints that CaM had a two domain structure with the N and C terminal parts independently folded [9, 10]. The first refined x-ray

Table 2. Amino acid sequence of some of the CaM-binding domains from various target enzymes

Enzymes	Amino acid sequences	K_d
Calcineunn	KEVIRNKIRAIGKMARVFSVLR	n.d.
skMLCK	KRRWKKNFIAVSAANRFKKISSSGAL	1 nM
smMLCK	RRKWQKTGHAVRAIGRLSSS	1 nM
PDE	TEKMWQRLKGILRCLVKQL	30 nm
Type II kinase	NARRKLKGILTTMLATRNFS	1 nM
CAM kinase	ARRKLKAAVKAWASSRLG	3–10 nM
Phosphorylase b kinase	LRRLIDAYAFRIYGHWVLGWWWNR	20 nM
Phosphorylase b kinase	GKGKVICLTVLASVRIYYWWVKP	6.5 nM
PF kinase	FMNNWEVYKIEARPAPKSGSYTV	11.4 nM
Ca^{2+} pump	RGWILWFRGLNRIWTWKVVNFSSS	600 nM
Spectrin	KTASPWKSARLMVHTVATFNSIKE	100 nM
B50/Neuromodulin	IQASFRGHITRKKL	4.2 μM
Adenylate cyclase	IDLLWKIAEAGARSAVG	580 nM
Caldesmon	GVRNIKSMWEKGNVFSS	0.8 μM
Calspermin	ARRKLKAAVKAVVASSRLGS	n.d.
Mouse hsp70	KRAVRRLRTACERAKRTLSSS	10 nM
$InsP_3$ kinase	EHYCLVRLMADVLRGCV	n.d.
MARCKS	SFKLSGFSFKKNKK	n.d.

determined crystal structure of CaM was reported by Babu *et al.* [11]. This structure showed a rather striking dumbbell shaped molecule, in which the N and C terminal regions were connected by a long α-helical structure, which was solvent exposed for several turns in the centre. The two domains are structurally homologous; each domain contained two calcium binding helix-loop-helix regions, and the two loops in each domain were connected to each other via three hydrogen bonds, thus forming a small antiparallel β sheet. The remainder of the molecule is to a large extent helical, with each calcium binding loop having a helix on either side. An interesting feature of the structure is that each domain has a hydrophobic surface patch, that contains 4 Met residues [11]. These residues comprise almost 50% of the surface area of these two homologous hydrophobic regions [8]. These surface regions were proposed to be involved in the binding of target proteins, and this notion has been supported by the subsequent determination of the structures of CaM complexed with target peptides (see below). We note with interest that these two hydrophobic surfaces are only present after Ca^{2+} is bound to the protein [12], thus explaining why the CaM mediated response can be calcium-dependent. Recently other x-ray structures for homologous CaM's have been reported, and all of these show the same structure [13–15]. However, the structure of Ca^{2+}-CaM has also been determined in solution by multidimensional NMR techniques [16], and in this study no evidence for an α helical structure in the central linker region was found. Subsequent NMR dynamical studies showed that this region was flexible, and allowed the two domains of CaM to move more or less independent of each other [17]. This obvious discrepancy between the CaM structures determined by x-ray and NMR techniques has recently been explained when it was shown that the organic solvents that are necessary to obtain proper crystallization conditions for Ca^{2+}-CaM, induced an α-helical structure in the linker region [18].

Three structures of Ca^{2+}-CaM complexed with a target peptide have recently been reported [19–21]. One of these was determined by NMR [19], and the other two were done by x-ray crystallography. The structure determined for the MLCK target peptide complexes by NMR and x-ray are rather similar; they differ in some details, but this may be caused by the fact that the amino acid sequence of the target peptides was not identical (they were derived from skeletal muscle and smooth muscle MLCK, respectively). The most striking feature of the CaM-MLCK peptide complex is that the target peptide is bound in a completely α-helical structure; this is rather remarkable, given that the target peptide in aqueous solution is devoid of regular secondary structure. The target peptide has an amphiphilic structure, and the two domains of CaM are bound through their Met-rich surface regions to the hydrophobic regions of the target peptide. Interestingly, in the complex, the two domains of CaM virtually retain their structure as compared to Ca^{2+}-CaM in the absence of a target peptide.

The major difference between the complex and calcium-CaM is that the central linker has been extended into a flexible loop structure, which allows the two domains of CaM to adopt a different orientation. In doing so they create a 'tunnel' that almost completely engulfs the helical target peptide. There are at least 150 contacts made between CaM and the MLCK peptides, with hydrophobic van der Waals contacts accounting for 80% of the total, while the remainder are primarily electrostatic interactions between the basic residues of the peptide and sidechains of some negatively charged (Glu) surface residues on CaM. The complex has an overall globular shape, and only 20% of the surface of the α helical peptide remains solvent exposed. The structure has been described as two hands capturing a rope [19]. The structure determined for the complex of Ca^{2+}-CaM with the target peptide of CaM kinase II, shows very similar features [21]. However, in this case the α-helix of the bound peptide is shorter, and this structure can be accommodated by positioning the two domains of CaM differently from the MLCK complex. This reorientation can be accomplished by further unwinding of the central helix; this region is therefore also referred to as an 'expansion joint'. Our NMR studies on the structure of the CaM-bound caldesmon peptide [22] also implied that the two domains of CaM can be repositioned, so that they are optimally placed for the binding of the hydrophobic regions of this short amphiphilic helical peptide.

Peptide binding to calmodulin

In order to study whether a proteolytically derived or a synthetic peptide is a proposed CaM-binding domain, a range of different experiments are available to the biochemist. Conceptually the simplest experiments are the gel shift assay and the competition studies with CaM-activated enzymes [23]. An example of the former is shown in Fig. 1. Most calmodulin binding domains bind with high affinity to CaM ($K_D \approx 1$–10 nM, see Table 2). Hence the interaction between such a peptide and CaM is tight enough that their complex will be maintained during electrophoresis. The experiment is generally done in 4M urea, in order to eliminate any nonspecific interactions [23]. As can be seen in Fig. 1, a complex is formed between CaM and a synthetic peptide which encompasses the CaM binding domain of MLCK. Complex formation is accompanied by a significant band shift. By placing a fixed amount of CaM, but different amounts of the peptide in each well of the gel, it is also possible to determine the stoichiometry of binding; in this case it is 1:1, since no further changes are observed at higher peptide to CaM ratios (see Fig. 1). Because the bandshift is only observed in the presence of Ca^{2+} and the absence of EDTA, it is clear that the binding is cal-

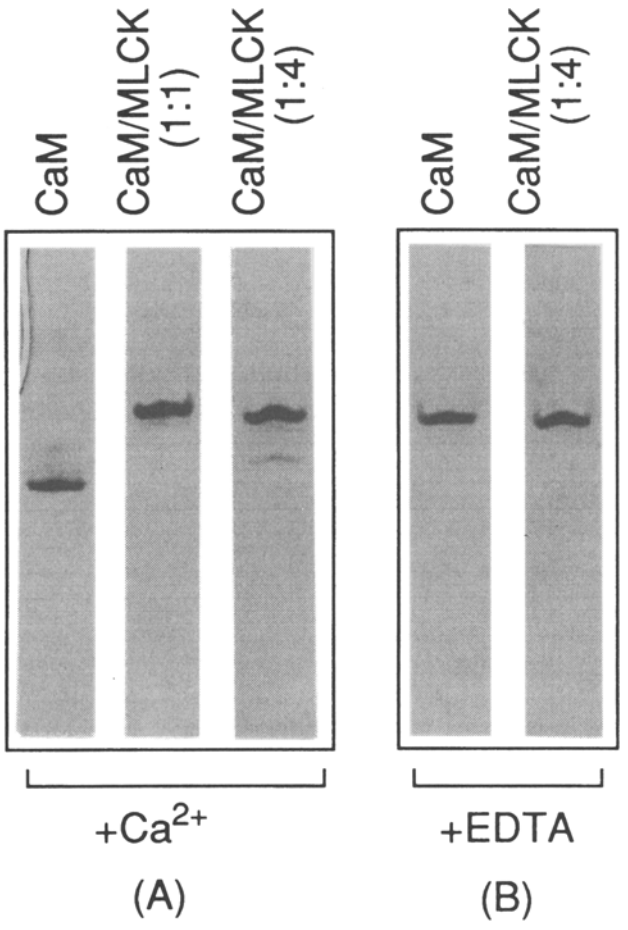

Fig. 1. Gel electrophoresis in 4 M urea of CaM in the presence and absence of Ca^{2+} and EDTA. Addition of the MLCK peptide gives rise to a bandshift with Ca^{2+}-CaM (A), but not for apo CaM (B). This effect is saturated at an equimolar ratio, suggesting that the stoichiometry of complex formation is 1:1. (For conditions see [23].)

cium-dependent, as is the activation of the enzymatic activity of MLCK. We have successfully used this procedure to demonstrate the interaction of a peptide with the proposed CaM-binding domain of constitutive Nitric Oxide Synthase to CaM [24]; also the CaM-binding domain of adenylate cyclase could be characterized in this fashion [25]. Moreover, we have also found that the model calmodulin binding peptide, melittin, binds to CaM in the same manner (Yuan and Vogel, unpublished observations). However, this gel shift experiment has not always been successful in our hands; aggregation properties of the synthetic peptides from other calmodulin-binding domains can sometimes lead to more complex gel patterns; likewise peptides that bind rather weakly to CaM will not be detected in this manner.

All the CaM-binding domains of the various target proteins studied to date bind to the two hydrophobic surface areas on CaM. While there appear to be differences in the details of their binding (*vide supra*), their binding sites on CaM overlap sufficiently, such that binding of one target protein prevents the activation of a second one. This property can be used to determine the relative K_D of a CaM binding peptide, as compared to the known K_D for a target enzyme [23]. For example, careful titrations with the cNOS CaM binding domain peptide, in competition experiments in the cyclic nucleotide phosphodiesterase assay allowed the determination of the K_D of the complex of its CaM complex [24]. Likewise peptides derived from the membrane spanning glycoprotein of the AIDS-causing human and simian immunodeficiency virus inhibited target enzyme activation by CaM [26, 27]. These data suggest that CaM may even be involved in the pathophysiology of this debilitating disease. We have recently shown that a 20 residue synthetic peptide encompassing the proposed CaM-binding domain of inducible Nitric Oxide Synthase markedly inhibits the activation of smooth muscle MLCK in a concentration dependent manner; such results support the notion that this region is indeed the CaM binding domain of the inducible NOS enzyme (unpublished results).

Another approach that has been successfully used relies on a column of immobilized calmodulin. Such affinity matrices have been produced primarily by attaching CaM through some of its lysine residues to a solid support. In such a column, CaM generally retains its ability to expose hydrophobic surfaces in a calcium dependent manner. Consequently this provides an elegant way through which one can purify a peptide containing the CaM binding domain, from a mixture of peptides generated by proteolytic or cyanogen bromide cleavage of an intact protein; this domain will bind in the presence of Ca^{2+}, and should be released upon elution with a calcium chelator such as EGTA [23]. However, since proteolytic cleavage can occur in the middle of a CaM-binding domain this approach does not guarantee success.

A final method used to establish binding between a peptide and CaM utilizes the intrinsic fluorescence of the Trp residues that are found in many of CaM-binding domains. Upon binding to the hydrophobic regions of the protein, the Trp residue will experience a blue shift as well as an increase in fluorescence intensity [28]. Because CaM itself is devoid of Trp residues, this approach provides a convenient way to follow the binding of a peptide; in fact, some investigators have used synthetic peptide analogs of CaM-binding domains in which a Phe has been replaced by a Trp to study binding [29]. The opposite approach has been used as well, where either a fluorescent probe [30], is covalently incorporated in the protein, or a Trp residue has been introduced into CaM by protein engineering methods [31]. While such studies can provide useful information about binding events, irrespective of the system studied, the introduction of spectral reporter groups always necessitates careful control experiments, which are required to demonstrate that the newly introduced group does not alter the system significantly.

Studies of the calmodulin interaction surfaces

As mentioned earlier, the two hydrophobic interaction surfaces on calmodulin are remarkably rich in Met residues. These regions have been called the 'methionine puddles', and 46% of the accessible surface areas of these two regions are provided by the Met sidechains [8]. It is therefore not surprising that many nOe contacts (distances < 5Å) could be found between the MLCK peptide and the Met sidechains of calmodulin, in an NMR study of the complex [19]. Likewise the crystal structures show that the Met sidechains of CaM bind to the target peptide [20, 21]. Another prominent amino acid in CaM's interaction surface is phenylalanine, each domain contains several of these residues, and while they are less exposed than Met residues, they are likely to contribute to the binding of target proteins, as the many nOe contacts in the NMR studies of the MLCK peptide in the complex demonstrate. We note with interest that preferential interactions between the sidechains of Phe and Met have been observed in many proteins [32], with the sulfur atom of Met lying in the plane of a Phe ring. It is quite possible that such preferred interactions also play a role in forming, maintaining and adapting the hydrophobic surface areas in calcium-CaM, particularly when a target peptide binds. Be that as it may, the sulfur atom of the Met sidechain has endowed this amino acid with two unique properties, flexibility and polarizability, that are not present in any aliphatic amino acid sidechain [33]. Firstly, because of the longer C-S bond length the energy barriers for rotations around these two bonds in the Met sidechains are much less than for a C-C bond; this creates a sidechain with unique flexibility that should make CaM's hydrophobic surfaces fairly adaptable to bind to hydrophobic areas of many shapes and forms. Secondly, the sulfur atom, in contrast to a carbon atom, is rather polarizable, meaning that it can alter the electron distribution in the Met sidechain to some extent. This may make it possible for the two hydrophobic surfaces of calcium-CaM to interact favourably with the polar solvent H_2O as well as interact with the apolar sidechains of a target peptide. It has also been suggested that the latter feature may enhance the London dispersion forces that stabilize the hydrophobic interactions [33].

Because of the importance of the Met sidechains for the function of CaM, they have been studied in considerable detail using a variety of approaches. The earliest study that drew attention to their possible involvement, were chemical modification studies showing that oxidation of Met abolished CaM's capacity to activate phosphodiesterase [34]. However, it was also shown in the same study that this modified protein had lost the ability to bind four calcium ions, suggesting that the modification lead to extensive conformational changes in CaM, thus complicating the interpretation of these data [35]. Subsequently, attention was drawn to the role of the Met sidechains by the large shifts observed in NMR studies upon binding calcium antagonist drugs, such as trifluoperazine [36]. While NMR chemical shift changes do not provide conclusive evidence about direct involvement, the protection of 8 Met residues against oxidation by H_2O_2 afforded by binding of a target peptide did (Huque and Vogel, unpublished results). As a result we decided to study CaM which was selectively labelled with ^{13}C-isotopically labelled terminal Met methyl groups (see Fig. 2). The NMR resonances were all assigned by making site-directed Met → Leu mutants of CaM. This approach afforded the assignment in many different forms of CaM, which allowed us to compare apo, Ca^{2+}, and various target peptide bound forms. NMR relaxation measurements were used to characterize the mobility of the Met methyl groups; as expected, these demonstrated a high degree of flexibility of these groups particularly in the uncomplexed calcium form [67]. Also, large chemical shift changes of these residues were observed upon binding of the Caldesmon, cNOS, Melittin, MLCK and PDE CaM binding domain peptides, further illustrating their direct involvement in binding.

In order to study the role of the polarizability of the sulfur atom, another NMR approach was developed. Because sulfur is nearly impossible to detect by NMR, and selenium-77 NMR is quite feasible, we substituted the amino acid analog selenomethionine for Met [37]. Moreover, while Se is slightly larger than S, it has a very similar polarizability, thus this substitution was not expected to alter the protein. In particular, when indirect detection of the Se atom via the more sensitive protons on the methyl groups proved possible (see Fig. 3), we

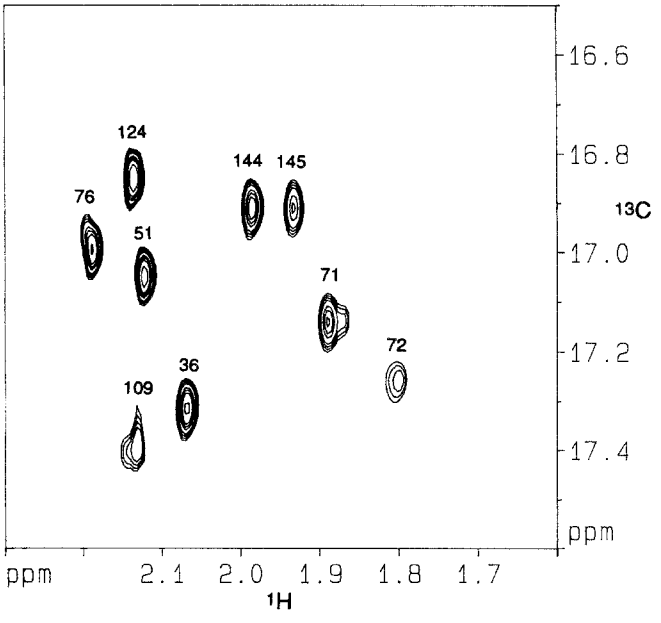

Fig. 2. Two dimensional ^1H, ^{13}C HMQC (Heteronuclear Multiple Quantum Coherence) NMR spectra of ^{13}C methyl Met selectively labelled CaM (55°C). CaM contains 9 Met residues, and their resonances can be discerned readily in such spectra [67].

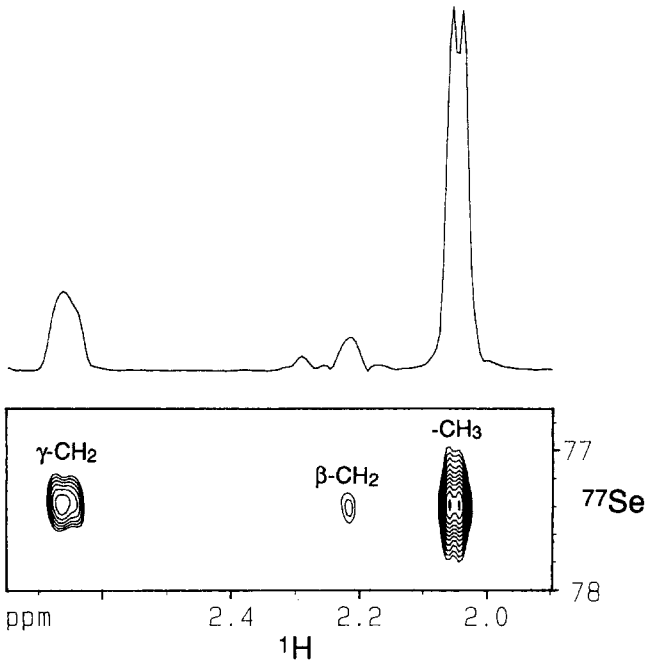

Fig. 3. Heteronuclear two dimensional ^{1}H, ^{77}Se correlation spectrum, obtained with the heteronuclear multiple bond correlation (HMBC) pulse sequence of the amino acid selenomethionine. Also the projection in the proton domain is plotted. Note that correlations from the Se atom to the CH_3 and the γ and β protons can be detected; however the correlation to the methyl group is much more intense and at natural abundance (^{77}Se, 7%) is the only one that can be reliably detected in a protein such as CaM [37].

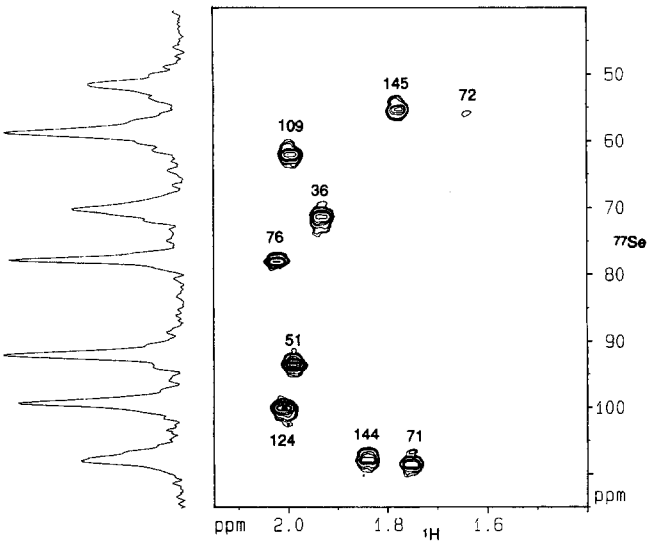

Fig. 4. Two dimensional HMBC spectrum of selenomethione Ca^{2+}-CaM showing the large chemical shift dispersion of the resonances along the ^{77}Se axis. A projection of the ^{77}Se spectrum is also 38 shown. This spectrum was recorded at 55°C. Note that all 9 Se Met resonances are detected, but that Met 72 has a much lower intensity presumably through some exchange process. 85% of the Met residues in this protein were replaced by SeMet, as shown by amino acid analysis [37].

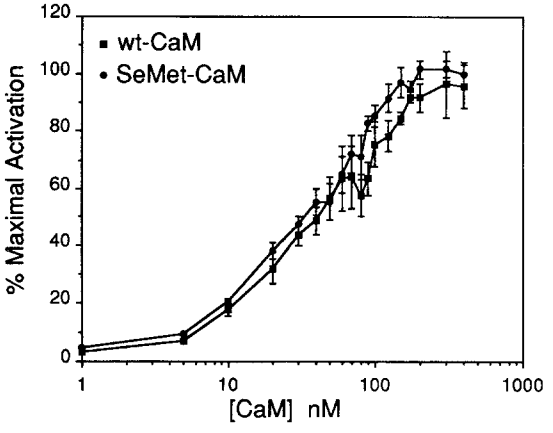

Fig. 5. Activation of smooth muscle Myosin Light Chain Kinase (MLCK) by SeMet-CaM and wild type CaM, showing similar activation properties of the two proteins.

could record two-dimensional spectra with excellent sensitivity for all the SeMet residues in calmodulin (see Fig. 4). Control experiments showed that the activation of PDE and MLCK by SeMet – CaM and wild type CaM were indistinguishable (see Fig. 5). We noticed that the ^{77}Se NMR chemical shifts of the SeMet residues in CaM were extremely sensitive to perturbations in the environment. Large shifts (up to 60 ppm) suggest that changes in the electron distribution around the Se atom (and by analogy the S atom) do indeed occur when calcium CaM binds a target peptide [37]. Thus, taken together, these NMR data support the notion that the unique flexibility and polarizability of the Met sidechains plays a role in the function of CaM.

Our next line of attack concerned studies of the activation of various target enzymes by the Met→ Leu mutants. To date, the activation of the enzymes phosphodiesterase [38], MLCK (Sutherland, Walsh and Vogel, unpublished results) and calcineurin (Edwards and Vogel, unpublished results) have been studied. All single and multiple Met → Leu mutants were also studied by FTIR and NMR spectroscopy, in order to confirm that they all had a structure similar to the native protein. For example, Fig. 6 shows part of the FTIR spectrum of the double mutant M36, 51L as well as the wild type CaM. Because their conformation sensitive amide I regions (around 1650 cm^{-1}) coincide almost perfectly, we conclude that the secondary structures of these two proteins are identical. Similar conclusions were reached for all of the mutant proteins, with the exception of M36L, which displayed a slight structural perturbation. Even a mutant protein, in which all four C-terminal Met residues were simultaneously replaced with Leu, appeared to have a normal structure [38]. Having established the correctness of the structure of the mutant proteins, we could be confident that any changes in enzymatic activity were caused directly by changes in the interaction interface. The results were rather unexpected; for example, M36L was nearly inactive with PDE, was fully active with MLCK, and activated Calcineurin to only 75%. In contrast M124L

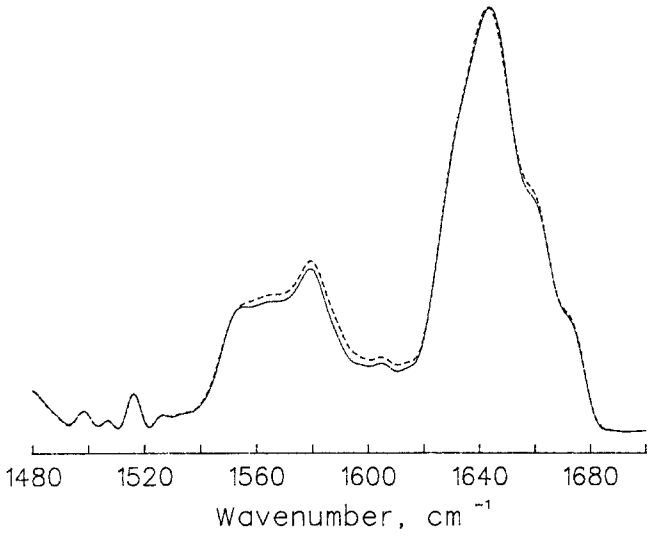

Fig. 6. Infrared spectra of wild type CaM (solid line), and the M36,51L double mutant (dashed line). The conformation sensitive amide I regions (around 1640 cm^{-1}) are identical, suggesting that the secondary structure is preserved in the mutant. The Asx, Glx region between 1550–1580 cm^{-1} is also the same, as is the Tyr (1517 cm^{-1}) and Phe (1478 cm^{-1}) aromatic region.

activated MLCK only 50% and required elevated protein concentrations, while it activated PDE and calcineurin like the wild type protein. These data underscore the notion that, while these three target enzymes share the same general binding site on CaM, there are great differences in the details of their binding.

Our present studies regarding the interaction surfaces of CaM have shifted towards the Phe residues. These are being studied by ^{19}F NMR of biosynthetically incorporated fluorophenylalanine residues (David, McIntyre and Vogel, unpublished observations). The rationale for this approach is twofold; first of all a convenient ^{19}F NMR probe is introduced in this fashion, and secondly the strongly electronegative fluorine atom should alter the electron distribution in the phenyl ring, and hence its interaction with other amino acid sidechains. Our preliminary data indicate that the substitution of ~50% F-Phe for Phe does not have a drastic effect on the structure or the PDE and MLCK activation properties of CaM.

Characterization of the calmodulin linker region

As stated before, the region connecting the two domains of calcium, CaM, appears as an α-helical structure in the available x-ray crystal structures. Nevertheless the ϕ and ψ angles for this part of the structure deviate significantly from a perfect helix, moreover the crystallographic B factors are high in this region [11]. Also, the available NMR and other spectroscopic data are consistent with the notion that the region from residue 77–82 is flexible in solution [17, 39]. The agreement between the outcomes of the NMR and x-ray analyses for the complex with the MLCK peptide is somewhat better; in solution the region from 74–82 appears disordered, while in the crystal structure the region from 73–77 has non α-helical angles and a high crystallographic B factor [19, 20]. In the crystal structure of the complex of CaM with the CaM kinase II peptide the region between residues 73 and 83 is totally disordered, as evidenced by the absence of electron density in this region [21]. This unravelling of the central linker is the most important change in the structure upon target peptide binding; at the same time, the structures of the two domains of CaM are virtually retained upon complex formation. Thus it would be useful to obtain more spectroscopic information about the status of the flexible linker region of CaM in the presence and absence of various target peptides. This could, for example, be done by measuring the NMR relaxation rates, and the hydrogen exchange rate for this part of the molecule, as was done for calcium-CaM. Such data have not been reported to date, but will undoubtedly become available in the near future.

We have used two different approaches to gather further information about the dynamics of the central linker of CaM. As indicated in the previous section, the Met residues of CaM have been studied by ^{13}C as well as ^{77}Se NMR. In both instances we found that Met76, which is located in the 'expansion joint' region of CaM is not affected by the binding of a peptide, or by the removal of Ca^{2+}. The ^{77}Se chemical shift is identical to that of the free amino acid SeMet in solution [37]. In fact, Met76 is the only Met residue that can be oxidized to a sulfoxide upon the binding of the model peptide melittin [40]. These observations are consistent with the idea that this region of the protein remains flexible and solvent exposed under all circumstances. We have also probed the environment of the Lys75 and Lys77 residues, by determining their sidechain pKa values in apo-, calcium, and target peptide bound (MLCK and cNOS peptides) forms [41–43]. We have found that Lys77 has a normal pKa value, expected for a fully exposed noninteracting Lys sidechain, in all three states of calmodulin. However Lys75 shows a totally different behaviour, it has a much reduced pKa value in apo- and calcium CaM; only after the binding of the target peptides does it obtain a pKa which is normal for an exposed surface Lys residue. The lower pKa for Lys75 can be explained by the interaction of its sidechain with the hydrophobic surface area of the N-terminal domain, which is clearly seen in the structure of the protein [11]. It could be stabilized by partial electrostatic interactions (for a discussion of such interactions see [44]) in this orientation. Once the target peptide is bound, the sidechain of Lys75 is forced away from this position, and now becomes fully exposed to the solvent. The position of Lys75 on top of the domain may, in fact, stabilize the helical conformation for this part of the linker, hence our data are con-

sistent with the NMR solution structure of calcium CaM in which K75 is fixed and forms part of helix IV. Upon binding the MLCK peptide the sidechain of K75 becomes part of the disordered loop structure in both the solution and crystal structure of the MLCK complex, this is consistent with our data suggesting that K75 is being forced to a solvent exposed position under these circumstances. In fact, the pH titration behaviour of K75 in a complex with the cNOS peptide [45], as well as the reactivity changes of K75 for the MLCK-CaM and calcineurin-CaM complexes [46, 47], show that K75 undergoes similar changes with these three proteins. Thus it is quite possible that the displacement of the K75 sidechain upon target peptide binding functions as a trigger, which contributes to the unwinding of part of the central helix into a loop structure. Interestingly K75 is highly conserved in CaM, suggesting that this trigger function may have been preserved throughout evolution.

The bound structure of the calmodulin-binding domain

The three presently available structures for complexes of CaM with target peptides, show that the calmodulin binding domains bind with a completely α-helical structure [19, 22]. This result was not completely unexpected, as many earlier circular dichroism studies of model peptides had indicated that these bound with an α-helical conformation to calmodulin [48]. An example of a CD study of CaM with the target peptide derived from MLCK is shown in Fig. 7. These data illustrate that the peptide in aqueous solution does not possess any regular secondary structure; the protein calcium CaM does, however, show a clear α-helical structure. Upon formation of the complex a further increase in α-helicity is observed. This is generally interpreted as indicating that the peptide binds to CaM in an α-helical conformation. However, it is very difficult on the basis of these CD data alone, to draw this conclusion. An alternative explanation of these data could be that the α helicity of CaM increases upon the binding of the peptide, and that the latter binds with an extended structure; such a mode of binding would also be consistent with the observed changes in the CD spectrum. These conflicting interpretations are difficult to resolve by CD spectroscopy alone because the bands for the α-helical structures in the protein and the peptide overlap. Fourier Transform Infrared (FTIR) Spectroscopy has emerged in the last decade as an alternative spectroscopic measurement for the determination of α-helical and β sheet structures in proteins and peptides [49, 50]. However, in FTIR Spectroscopy the characteristic bands for a protein and a peptide in a complex would also normally overlap. Nevertheless, by complete carbon-13 isotope labelling of the protein, it is possible to shift the amide I band, which is sensitive to secondary structure formation, by a wavenumber of ≈ 50 cm^{-1} [51, 52].

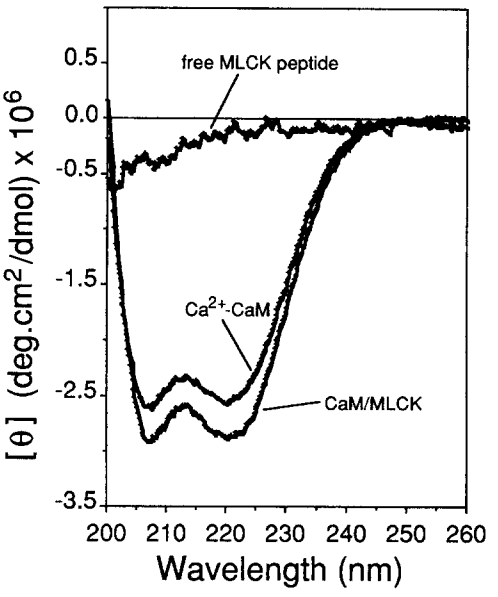

Fig. 7. Circular dichroism spectra (far ultraviolet) of the MLCK peptide, Ca^{2+}-CaM and their complex. Note the increase in α helix content upon complex formation (α-helices give a double minimum at 208 and 222 cm^{-1}).

We have applied this approach to CaM, and the FTIR spectrum of the isotopically labelled and unlabelled protein are compared in Fig. 8. The amide I band of calmodulin is shifted 55 cm^{-1} to lower wavenumber, leaving a clear window in the spectrum around 1640–1660 cm^{-1}, where the amide I band of the target peptides that are bound to CaM resonate. We have successfully used this approach to demonstrate that synthetic peptides containing the CaM-binding domains of MLCK, cNOS and caldesmon bind to CaM with an α-helical conformation [52]. Interestingly, difference FTIR spectra also showed that the changes in the secondary structure of CaM upon complex formation are minimal, which is in agreement with NMR and x-ray studies of the complexes [19–22, 53]. Our results demonstrate that 'isotope-edited' FTIR experiments provide a useful means of studying such protein-protein and protein-peptide interactions. While these CD and FTIR data can show that the CaM bound peptides are primarily α-helical, they do not indicate what exact regions of the peptide take part in the α-helix formation. In order to determine this, one can solve the structure of the bound peptide by NMR methods.

The majority of CaM-binding domains bind rather tightly to CaM with K_D's on the order of nM. Consequently, the CaM-bound and free peptide will be in slow exchange on the NMR timescale. Under such conditions one can solve the structure of the bound peptide in the complex, by synthesizing a totally ^{15}N or ^{13}C labelled peptide and allowing this to bind unlabelled CaM [54]. By applying suitable isotope filters in the two- and three-dimensional NMR experiments it is then possible to suppress all the NMR signals for the

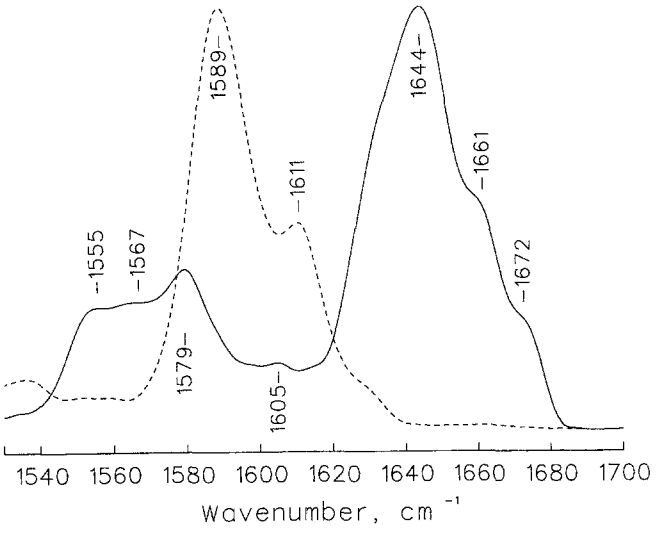

Fig. 8. Infrared spectra of wild type CaM (solid line) and totally 13C labelled CaM (dashed line). Note the large shift of the amide I peak from 1644 to 1589 cm^{-1}. Peaks for CaM bound target peptides appear between 1640 and 1660 cm^{-1} and can be studied without overlap when totally (> 99%) isotopically labelled CaM is used [52].

protons bound to the ^{14}N and ^{12}C nuclei of the protein, and only observe those bound to ^{15}N and ^{13}C in the bound peptide. This approach has allowed Roth *et al.* [54] to demonstrate conclusively that the MLCK peptide bound to CaM in an α helical manner. However, the cost involved in the solid phase synthesis of an isotopically labelled peptide is rather high, while the cost of totally biosynthetic labelling of a protein by feeding $^{15}NH_4Cl$ and $^{13}C_6$-glucose to a bacterial expression system is considerably less in most instances. Hence, Ikura and Bax [55] performed the opposite experiment in which all ^{15}N and ^{13}C bound proton signals were suppressed, and only those for the ^{14}N and ^{12}C in the unlabelled MLCK peptide were observed. Their work gave the same α-helical structure for the bound peptide, as observed earlier by Roth *et al.* [54]. Ultimately these elegant isotope filtering and multidimensional NMR approaches also allowed Ikura and coworkers [19] to determine the structure of the CaM-MLCK peptide complex, which is a significant accomplishment given the size of the complex.

Some target peptides that bind with a lower affinity (1 μM or higher) to CaM are more easily studied by the so-called transfer Nuclear Overhauser Effect (trnOe) NMR experiment. This involves studying the target peptide in the presence of a small amount of the protein. Because free and bound peptide are now continuously exchanging, at a fast to intermediate rate on the NMR timescale, the peptide resonances in solution will still have a 'memory' of their bound structure [56]. This allows one to determine the structure of the bound peptide in a straightforward manner from the resonances for the peptide in solution; for example, we have shown that the central 10-residues of the caldesmon calmodulin binding domain form an α-helical structure upon binding to CaM, while the ends of this target peptide remain extended [22]. Also, in the same manner we have been able to demonstrate that the calmodulin binding domain of neuromodulin/B50, which binds preferentially to the apo-CaM, does so with an α-helical structure [56]. However, no information about the location of the binding site is gleaned from the trnOe experiments, and to obtain such information requires various NMR titration experiments to follow perturbations in chemical shift [22]; in addition, paramagnetic broadening experiments with nitroxide spin labelled proteins or peptides [45] can be done in order to map out the binding site and the orientation of the peptide.

Since the majority of CaM-binding peptides are rather hydrophobic in nature, their solubility, or the solubility of their complexes with CaM in aqueous solution can sometimes be a problem at the concentrations required for NMR studies (0.5–2.5 mM). In such instances we have found that most of these peptides can be dissolved by adding trifluoroethanol, a well known helix stabilizing solvent, to the aqueous solution; the same solvent may also stabilize β turns. Since both calmodulin and TFE appear to induce α-helical structures in linear peptides, although not necessarily through a similar mechanism, this solvent system is very useful in studies of these particular peptides [58]. CD studies require much lower protein concentrations than NMR; under these conditions aggregation is often less of a problem and hence CD titration experiments are useful to follow the conformational transition of a largely unstructured peptide to an α helical peptide as the concentration of trifluoroethanol is increased [22, 24, 58]. For most peptides we have studied to date, this transition is complete when 40% TFE (v/v) is added.

The α-helical structure of the target peptide can then be determined by NMR in aqueous TFE solution. An example of such a study is shown in Figs 9 and 10. The first step is the acquisition of a two-dimensional NOESY NMR spectrum, which together with a total correlation (or TOCSY) spectrum is needed to obtain the sequential assignment of all the peptide resonances [59]. Once this task has been completed an inspection of the nOe patterns provides information about the secondary structure formation. For example, in an α-helical structure the αNH protons of adjacent amino acids are within 5 Å of each other and should give rise to a short range nOe effect (d_{NN} (i, i + 1)). Because each turn of an α-helix involves 3.6 amino acid residues, parts of the residues i and i + 3, are placed more or less on top of each other, thus giving rise to medium range nOe effects ($d_{\alpha\beta}$ (i, i + 3), $d_{\alpha N}$ (i, i + 3)). This pattern of short and medium range nOe's is very diagnostic for α-helix formation [59], as the same nOe interactions are not present in a β sheet or an extended structure. Fig. 8B shows the amide region of the NOESY spectrum of the cNOS peptide acquired in 40% TFE and reveals the presence of many intense d_{NN} connectivities;

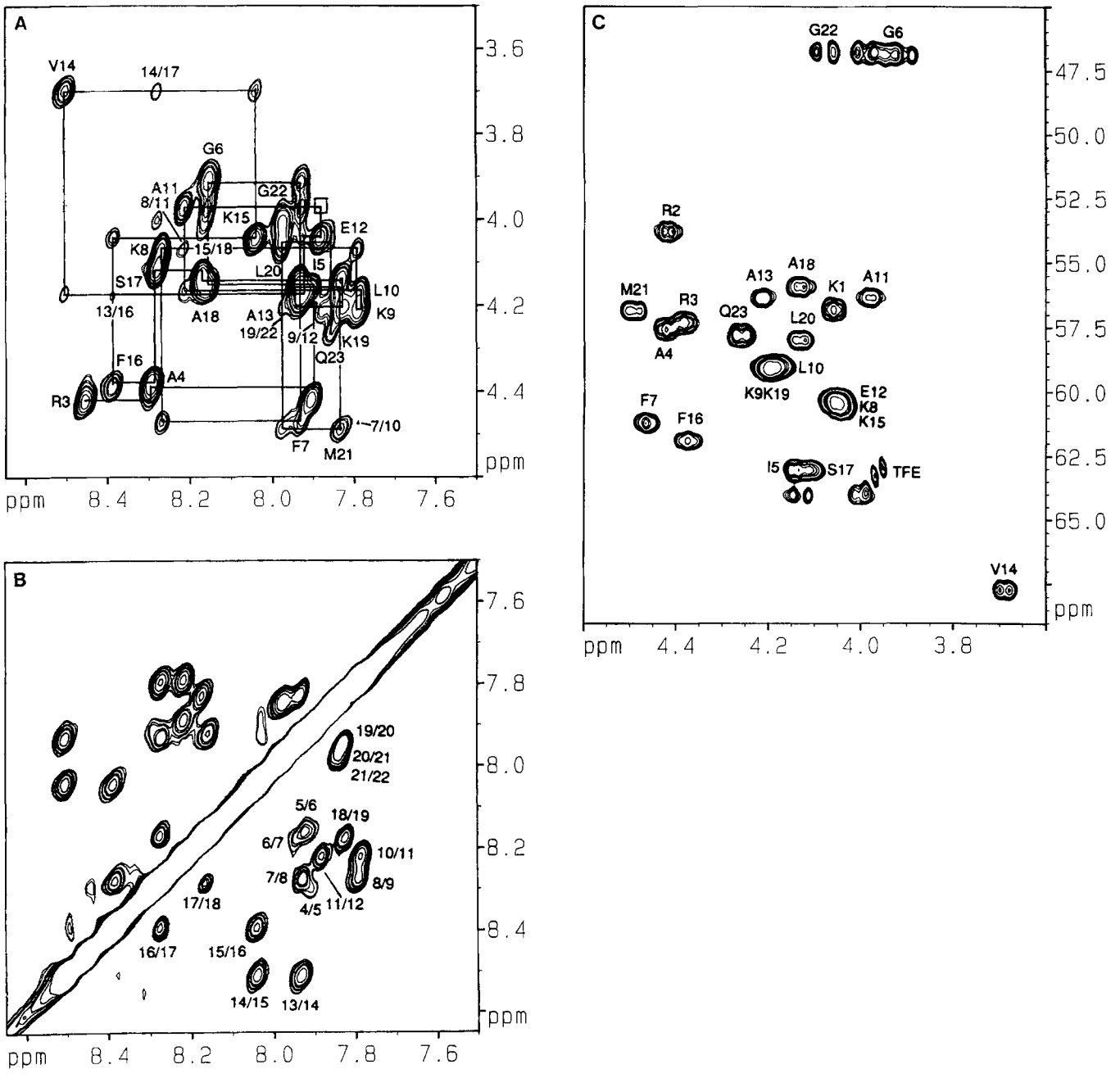

Fig. 9. Two dimensional NMR spectra of the cNOS peptide recorded in 40% TFE (for amino acid sequence see Fig. 10). A) Fingerprint region of the NOESY spectrum (mixing time 200 ms). B) Amide region of the NOESY spectrum. C) Part of the ^{1}H, ^{13}C Heteronuclear Multiple Quantum Coherence (HMQC) spectrum showing correlations between the αH and αC resonances. The numbers correspond to the amino acid residue numbers.

Fig. 8A provides the sequential assignment that are traced out through the $d_{\alpha N}$ (i, i + 1) connectivities, and also shows the presence of many $d_{\alpha N}$ (i, i + 3) crosspeaks in the fingerprint region of the NOESY spectrum. The nOe data are subsequently summarized as indicated in Fig. 10; from this Table it is clear that an α helical structure can be formed from Ile-5 all the way to Met 21 in the peptide.

Further evidence for the presence of an α-helix can be obtained by studying the chemical shifts of the αH resonances, which are expected to shift upfield [60] when they are involved in an α-helical structure; indeed this pattern is found as depicted in the middle panel of Fig. 10. Conversely the chemical shifts for the α carbon atoms are known to shift substantially downfield if they are involved in an α-helix [60, 61]. In order to obtain these ^{13}C NMR data it is necessary to obtain a proton carbon heteronuclear correlation spectrum as shown in Fig. 9C; these can be acquired with reasonable sensitivity using the natural abundance (1.1%) of carbon-13. The

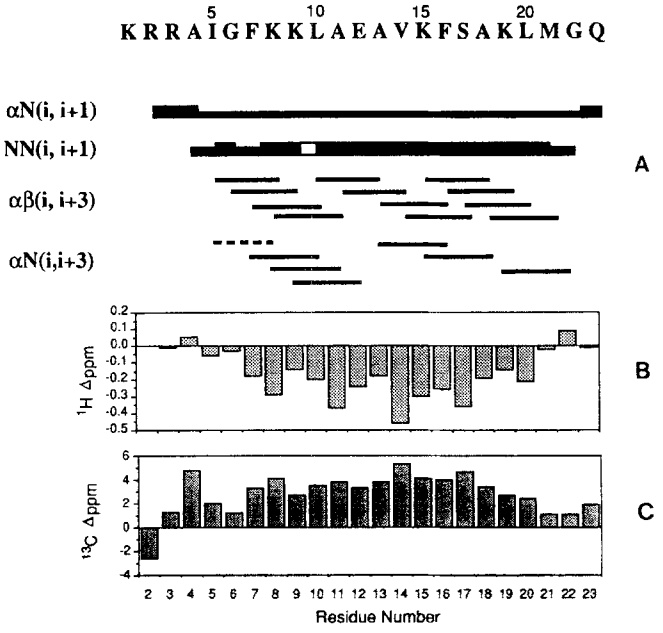

Fig. 10. Amino acid sequence and summary of the structural NMR data for the cNOS peptide in 40% TFE. A) The various nOe interactions observed for the cNOS peptide. The thickness of the lines corresponds to the nOe intensities. B) The ^{1}H NMR chemical shift difference for the aH resonance in the cNOS peptide compared to shifts measured in a random coil peptide. C) The ^{13}C NMR chemical shift difference for the αC resonances in the cNOS peptide compared to shifts measured in a random coil peptide.

bottom panel in Fig. 10 shows that the ^{13}C α carbon shifts also show the expected downfield shifting behaviour. With all these data in hand, the presence of an α-helix between Ile-5 and Met 21 can be identified without ambiguity. It is noteworthy that the αH and αC chemical shift distributions appear with an almost Gaussian distribution curve; this suggests that the helix is most stable in the centre, while the ends can unfold and refold (a phenomenon referred to as fraying). NMR titration and paramagnetic broadening experiments discussed above can then be used in combination with molecular modelling and mechanics calculations to dock the α-helical peptide onto calmodulin.

While the structures of all the CaM bound target peptides studied to date appear to be α-helical, this does not imply that these CaM-binding domains also have an α-helical structure as part of the larger target protein. It is quite likely that the induction of the α-helicity is dictated by the binding of these domains to CaM. Unfortunately very few structural details are known about the various target proteins, and it would be interesting to determine the structure of such a protein in the absence and presence of CaM. Such a study would in all likelihood have to be done by x-ray crystallography, because all the physiologically interesting target proteins are too large to be studied by NMR methods. At present, only a modeled structure for part of the CaM-binding domain of MLCK is available [58]. Interestingly, this domain has an extended structure when it is part of MLCK. This would suggest that a large change in the secondary structure of the domain occurs upon binding to CaM; in all likelihood, this conformational change contributes to the activation of the target protein (for further discussion see [58]).

Epilogue

Calmodulin represents an unusual case of protein-protein or protein-peptide interactions. Such interactions have been studied extensively for immunoglobulins, proteolytic enzymes and also for some protein kinases and phosphotyrosine binding SH2 domains (for a recent review see [62]). In nearly all these instances peptides bind in an extended conformation, and interactions occur between sidechains, as well as with backbone functional groups. This mode of binding is believed to give rise to high specificity and very selective interactions. In contrast, calmodulin is a rather promiscuous protein that interacts with many different peptides that do not share any sequence homology. The low specificity and selectivity of CaM's interactions with its many partners can now be related to two features. First, the binding of a peptide to the two methionine rich surfaces induces an α helical conformation, and interactions only take place through amino acid sidechains; the intrinsic flexibility and polarizability of Met makes these two surfaces highly malleable and sticky, so that peptides with many different sequences can be accommodated. Secondly, the flexibility of the linker region allows the protein to adjust the orientation of the two domains with respect to each other and the target peptide, so that amphiphilic peptides of different length and with a different distribution of hydrophobic groups can be accommodated. Clearly, our understanding of CaM-target protein interactions has increased considerably since we last reviewed this topic [63].

Now that these features of CaM are becoming more established one can ask whether we fully understand this intriguing protein. We feel that we are only just beginning to understand this important calcium regulatory protein. Many questions still need to be answered. For example, to what extent is the binding of CaM to a CaM-binding domain transmitted throughout an entire target protein? Such studies would require the analysis of an entire CaM target protein complex, rather than the CaM-target peptide structures determined to date. Not all target proteins bind with a single CaM-binding region to CaM, an example of such a protein is phosphorylase kinase [64], and it would be interesting to study the structure of such an obviously more complex system. Furthermore, some target proteins (such as iNOS) bind to CaM even in the absence of Ca^{2+}, again it would be interesting to determine whether the structure of such a complex resembles that of the ones determined to date. A structure for the apo form of CaM is needed, in order to understand how

this form shields the interaction surfaces from the solvent and peptide binding. While CaM has been known for some time to play a role in gene expression and cell cycle control [65], only recently have unique DNA-binding proteins been identified which appear to be nuclear targets for calmodulin [66]; we can now begin to explore the mechanism of action of CaM in gene expression. Most cells contain multiple targets for CaM, and the concentration of CaM is generally too low to bind to and activate all of them simultaneously; hence there is a need to find out how the different target proteins are selected both in a spatial and temporal manner to achieve the proper cellular response. Finally one wonders if it will be possible to produce mutant CaMs which have more selective activation properties. Our data obtained with Met → Leu mutants to date suggests that this may be the case. Transfection of such mutants into specific cells will be a useful experimental tool, and such mutant proteins could potentially have future medical applications.

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