Eur Biophys J (1997) 25: 239–247

ARTICLE

Tudor Porumb · Anna Crivici · Perry J. Blackshear
Mitsuhiko Ikura

Calcium binding and conformational properties of calmodulin complexed with peptides derived from myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS-related protein (MRP)

Received: 26 September 1996 / 22 October 1996

Abstract The myristoylated alanine-rich C kinase substrate (MARCKS) and the MARCKS-related protein (MRP) are members of a distinct family of protein kinase C (PKC) substrates that bind calmodulin (CaM) in a manner regulated by Ca^{2+} and phosphorylation by PKC. The CaM binding region overlaps with the PKC phosphorylation sites, suggesting a potential coupling between Ca^{2+}-CaM signalling and PKC-mediated phosphorylation cascades. We have studied Ca^{2+} binding of CaM complexed with CaM binding peptides from MARCKS and MRP using flow dialysis, NMR and circular dichroism (CD) spectroscopy. The wild-type MARCKS and MRP peptides induced significant increases in the Ca^{2+} affinity of CaM (pCa 6.1 and 5.8, respectively, compared to 5.2, for CaM in the absence of bound peptides), whereas a modified MARCKS peptide, in which the four serine residues susceptible to phosphorylation in the wild-type sequence have been replaced with aspartate residues to mimic phosphorylation, had smaller effect (pCa 5.6). These results are consistent with the notions that phosphorylation of MARCKS reduces its binding affinity for CaM and that the CaM binding affinity of the peptides is coupled to the Ca^{2+} affinity of CaM. All three MARCKS/MRP peptides perturbed the backbone NMR resonances of residues in both the N- and C-terminal domains of CaM and, in addition, the wild-type MARCKS and the MRP peptides induced strong positive cooperativity in Ca^{2+} binding by CaM, suggesting that the peptides interact with the amino- and carboxy-terminal domains of CaM simultaneously. NMR analysis of the Ca^{2+}-CaM-MRP peptide complex, as well as CD measurements of Ca^{2+}-CaM in the presence and absence of MARCKS/MRP peptides suggest that the peptide bound to CaM is non-helical, in contrast to the α-helical conformation found in the CaM binding regions of myosin light-chain kinase and CaM-dependent protein kinase II. The adaptation of the CaM molecule for binding the peptide requires disruption of its central helical linker between residues Lys-75 and Glu-82.

Key words Calmodulin · MARCKS · Calcium binding · NMR

T. Porumb · A. Crivici[1] · M. Ikura (✉)
Division of Molecular and Structural Biology,
Ontario Cancer Institute and Department of Medical Biophysics,
University of Toronto, 610 University Avenue, Toronto,
Ontario, Canada M5G 2M9

P. J. Blackshear
Howard Hughes Medical Institute Laboratories and
Division of Endocrinology, Metabolism and Nutrition,
Departments of Medicine and Biochemistry,
Duke University Medical Center, Durham, NC 27710, USA

M. Ikura
Center for Tsukuba Advanced Research Alliance and
Institute of Applied Biochemistry, University of Tsukuba,
Tsukuba 305, Japan

Present address:
[1] Allelix Biopharmaceuticals Inc., 6850 Goreway Drive,
Mississauga, Ontario L4V 1V7, Canada

Introduction

The myristoylated alanine-rich C kinase substrate (MARCKS) and its homologue, the MARCKS-related protein (MRP; also referred to as F52 and MacMARCKS), are major cellular substrates for protein kinase C (PKC) (see Aderem 1992a, b; Blackshear 1993 for reviews). Both MARCKS and MRP are myristoylated at the amino-terminus and bind calmodulin (CaM) and actin, in both cases in a manner regulated by PKC phosphorylation. For example, it has been shown that MARCKS only binds CaM in the presence of Ca^{2+}, and that phosphorylation of MARCKS markedly decreases its affinity for Ca^{2+}-CaM. MARCKS also binds to the sides of the actin filaments and crosslinks them, and this activity is inhibited by either MARCKS phosphorylation or Ca^{2+}-CaM binding (Aderem 1992b). As such, MARCKS (and analogously MRP) may integrate Ca^{2+} signal and phosphorylation cascade pathways.

A conserved 24- or 25-amino acid residue sequence, located approximately in the middle of the MARCKS and MRP sequences, contains the CaM binding region, the PKC phosphorylation sites, and is also responsible for actin binding. Previous studies (Graff et al. 1989; McIlroy et al. 1991; Blackshear et al. 1992; Verghese et al. 1994) have shown that synthetic peptides, referred to as the MARCKS (tetra-Ser) and MRP peptides (Fig. 1), comprising this conserved sequence bind CaM. These peptides contain the two to four serines that are phosphorylated by PKC. These peptides have been shown to represent excellent models of the intact MARCKS and MRP proteins in terms of the interactions between CaM binding and phosphorylation by PKC (Verghese et al. 1994). The MARCKS (tetra-Ser) and MRP peptides bind Ca^{2+}-CaM with high affinity (~3 nM) (Graff et al. 1989; Verghese et al. 1994; McIlroy et al. 1991). Phosphorylation of the MARCKS (tetra-Ser) peptide by PKC has been shown to decrease its affinity for CaM by more than 200-fold (McIlroy et al. 1991; Blackshear 1993). The analogous peptide, referred to as MARCKS (tetra-Asp) (Fig. 1), in which the four "phosphorylatable" serine residues of the MARCKS (tetra-Ser) peptide are all substituted with an aspartate residue, partially mimics the properties of the phosphorylated peptide. Indeed, the MARCKS (tetra-Asp) peptide binds CaM with an approximately 10-fold lower affinity than that of the wild-type derived MARCKS (tetra-Ser) peptide (Hinrichsen and Blackshear 1993).

Although both MARCKS and MRP bind CaM with high affinity in a calcium-dependent manner, there is some evidence that these complexes may be atypical compared to those seen with other CaM binding peptides. For example, most other CaM binding sites have been shown to be α-helical (DeGrado 1988; O'Neil and De Grado 1990) and the CaM binding peptides of both MARCKS and MRP can be modeled as amphiphilic α-helices (Graff et al. 1989; Li and Aderem 1992). However, the MARCKS CaM binding domain has been shown by circular dichroism measurements to be a random coil in solution (Coburn et al. 1993), as predicted by secondary structure algorithms (Stumpo et al. 1989). In addition, the MRP CaM binding domain contains a proline not present in the MARCKS peptide, in a position that might be expected to disrupt an α-helix; yet the affinities of these two peptides for CaM are very similar (Verghese et al. 1994). These findings raise the possibility that the CaM complexes formed by MARCKS and MRP might be novel in conformation, and might also modulate Ca^{2+} binding by CaM in an unusual way.

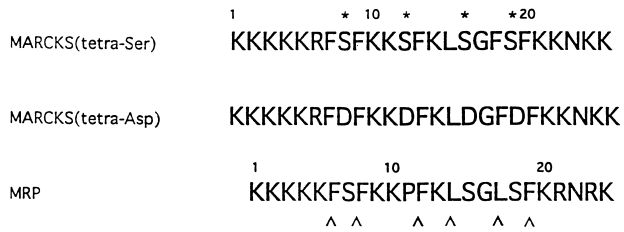


Fig. 1 Amino acid sequence of the homologous MARCKS/MRP peptides used in this study. The four sites of phosphorylation by PKC are designated by an *asterisk*. Hydrophobic residues are marked by ^

Experimental procedures

Protein purification and Ca^{2+} removal

Recombinant *Xenopus laevis* CaM (expression vector kindly provided by Dr. Claude Klee, National Institutes of Health) was expressed in *E. coli* and purified as described previously (Ikura et al. 1990). Uniformly ^{15}N-labeled and $^{13}C/^{15}N$-labeled CaM samples were prepared using M9 minimal medium containing $^{15}NH_4Cl$ and/or [$^{13}C_6$]-D-glucose as sole nitrogen and carbon sources, respectively (Ikura et al. 1990). The proteins were decalcified by trichloroacetic acid precipation and renaturation in the presence of 1 mM EGTA using the procedure described previously (Porumb et al. 1994). Residual Ca^{2+} concentration in the protein samples was less than $[Ca^{2+}]/[CaM] = 0.1$, as determined using atomic absorption spectrometry. Protein concentrations were determined from amino acid analysis.

The peptides

Three peptides, MARCKS (tetra-Ser), MARCKS (tetra-Asp), and MRP (Fig. 1), were synthesized and purified as described previously (Hinrichsen and Blackshear 1993; Verghese et al. 1994).

Calcium binding assay

The Ca^{2+} binding properties of CaM in the absence and in the presence of MARCKS/MRP target peptides were measured using the flow-dialysis apparatus described by Porumb (1994). The dialysis medium contained protein and peptide (Table 1) in 50 mM HEPES, 0.1 M KCl buffer, pH 7.5, at 25 °C. In the case of CaM, 1 mM $MgCl_2$ was supplemented to be consistent with previous work (Porumb et al. 1994; Maune et al. 1992; Starovasnik et al. 1993). The Ca^{2+} binding data were quantitatively analyzed using the classical Hill binding model (Dahlquist 1979; Porumb 1994). Nonlinear regression was performed using extensions of the Enzfitter computer program (Leatherbarrow 1987).

NMR

Uniformly ^{15}N- and $^{15}N/^{13}C$-labeled CaM were dissolved in 95% H_2O/5% D_2O, containing 0.1 M KCl, 4.5 mM $CaCl_2$, pH 6.2–6.8 for a final concentration of approximately 1 mM in a 0.5 ml volume. CaM-peptide complexes were prepared by direct addition of concentrated peptide solutions (typically 20 mM) to the NMR sample of labeled CaM.

Table 1 Ca^{2+} binding to CaM and its complex with MARCKS, MRP and skMLCK peptides

Sample	CaM/peptide concentration (μM)	K_d (M) (average)	Hill coeff.
(CaM[a]	40	7.2×10^{-6}	1.0)
CaM-MARCKS (tetra-Asp)	27/180	2.3×10^{-6}	0.92
CaM-MRP	15.3/70	1.5×10^{-6}	1.56
CaM-MARCKS (tetra-Ser)	16.2/70	9.0×10^{-7}	1.4
CaM-skMLCK[b]	5.4/70	2.6×10^{-8}	1.5

[a] The use of the simple Hill binding model in the case of free CaM is an over-simplification, as it is known that the C- and N-terminal domains of free CaM bind Ca^{2+} independently, with cooperativity between the two Ca^{2+} binding sites of each domain (Minowa and Yagi 1984; Klee 1988; Porumb 1994). However, the value is included for comparison. If independent Ca^{2+} binding to the two domains of CaM is assumed, the corresponding K_d values for the two domains are 2.4×10^{-6} M and 1.8×10^{-5} M, with respective Hill coefficients of 1.7 and 1.6 (Porumb et al. 1994).

[b] Data from Porumb et al. 1994

NMR spectra were recorded at 25 °C on a Varian UNITY-plus 500 spectrometer equipped with a four-channel, actively z-gradient shielded triple-resonance probe and a pulse field gradient (PFG) driver. ^{1}H-^{15}N HSQC spectra were obtained using the sensitivity enhanced method described by Kay et al. (1992). The ^{15}N-edited NOESY-HMQC, ^{15}N-edited TOCSY-HMQC, CBCA(CO)NH and HBCBCA(CO)CAHA spectra were obtained as described previously (Ames et al. 1994). $^{13}C/^{15}N$-filtered NOESY and COSY spectra were recorded using the pulse sequences described previously (Lee et al. 1994; Ikura and Bax 1992).

CD Spectroscopy

Spectra were recorded on a Jasco-720 spectropolarimeter using a 1-mm path length quartz cell equilibrated at 25 °C. Ten spectra, recorded from 200–250 nm, were collected for each sample and were averaged using J700 software provided by the manufacturer. Spectra were recorded using 10 μM solutions of Ca^{2+}-CaM-peptide complex prepared in 50 μM Tris, pH 7.5, and were corrected for the absorbance of the buffer medium. The data were expressed as mean residue ellipticity.

Results

Ca^{2+} binding properties of CaM in complex with MARCKS/MRP peptides

Ca^{2+} binding of CaM in the absence and the presence of MARCKS (tetra-Ser), MRP and MARCKS (tetra-Asp) peptides was measured using the flow-dialysis method. Binding of each of the three peptides to CaM induced significant shifts of the Ca^{2+} binding curve towards lower Ca^{2+} concentrations (Fig. 2). The largest enhancement (~8 fold) was observed for MARCKS (tetra-Ser), and the smallest change (~3 fold) was induced by MARCKS (tetra-Asp), while the MRP peptide displayed an intermediate change (~5 fold). Apparent dissociation constants and Hill coefficients are summarized in Table 1. The Hill coefficients obtained for CaM complex with MARCKS (tetra-Ser) and with the MRP peptide were much higher than the coefficient obtained for CaM complexes with MARCKS (tetra-Asp), and were similar to that reported for the CaM-skMLCK peptide complex (Table 1).

Structural features of the Ca^{2+}-CaM-MRP peptide complex

^{1}H-^{15}N HSQC spectra (not shown) were recorded for uniformly ^{15}N-labeled, Ca^{2+}-bound CaM with successive addition of MARCKS (tetra-Ser), MRP or MARCKS (tetra-Asp) peptides. During the early course of peptide titration, the ^{1}H-^{15}N HSQC spectra of Ca^{2+}-CaM in the presence of MARCKS (tetra-Ser) and MRP showed doubling of the cross peaks for a large number of backbone amide protons, suggesting that the exchange rates between CaM and peptide-bound CaM were in the slow-exchange limit of the NMR time scale. Such slow-exchange phenomena have been observed in the cases of the Ca^{2+}-CaM-skMLCK peptide complex, and are correlated with the strong association ($K_d \sim 1$ nM) between the skMLCK peptide and CaM (Takio et al. 1985). In contrast, the spectrum of Ca^{2+}-CaM in the presence of the MARCKS (tetra-Asp) peptide resulted in continuous changes of peak positions. This fast exchange behavior is consistent with the approximately 10-fold lower affinity of the MARCKS (tetra-Asp) peptide

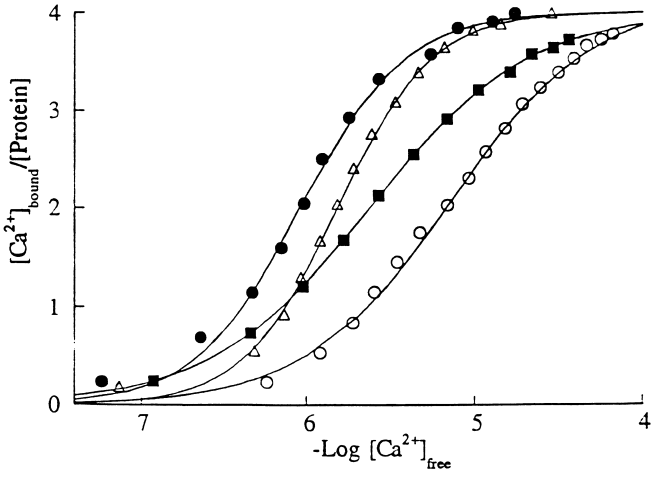


Fig. 2 Ca^{2+} binding curves for CaM (○) and CaM in complex with MARCKS (tetra-Ser) (●), MRP (△), and MARCKS (tetra-Asp) (■) peptides. The Ca^{2+} binding profiles were obtained by the flow-dialysis method and analyzed with the Hill binding model, as specified in Materials and methods and in Table 1. The *solid lines* represent fitted curves for each set of data

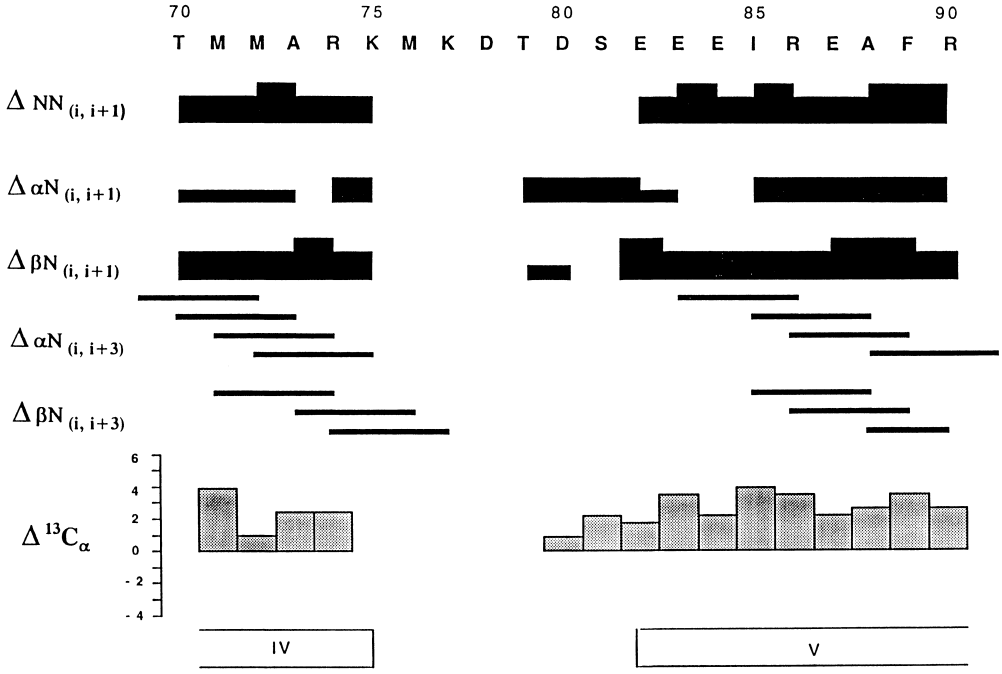


Fig. 3 Partial amino acid sequence of the CaM "central helix" region and a summary of NOE connectivities involving HN, $C^{\alpha}H$ and $C^{\beta}H$ protons is presented for the complex of Ca^{2+}-CaM with the MRP peptide together with the secondary structure deduced from these data. The differences between the $^{13}C\alpha$ chemical shift of each amino acid residue and the random-coil shift are also plotted as a function of residue number. The NOE intensities are indicated by the black bars and are classified according to the observed intensity of each cross peak in the NOESY-HMQC spectrum, determined qualitatively by the number of contour levels plotted in the two-dimensional spectra. Data for residues 76–78 are missing due to excessive broadening of the respective resonances and may indicate an ill-defined conformation in that region

for CaM compared to that of MARCKS (tetra-Ser) and the MRP peptide (Hinrichsen and Blackshear 1993).

With the aid of previously reported resonance assignments for Ca^{2+}-CaM complexes with the skMLCK peptide (Ikura et al. 1991), it was possible to assign most of the resonances for Ca^{2+}-CaM complexed with the MRP peptide using a combination of 3D ^{15}N-edited NOESY-HMQC and ^{15}N-edited TOCSY-HMQC experiments. The well-resolved resonances in the spectra were easily assigned by comparing the chemical shifts with those of the Ca^{2+}-CaM-skMLCK peptide complex. Further assignments were achieved on the basis of short-range NOE connectivities such as strong $NN_{(i,i+1)}$, weak $\alpha N_{(i,i+1)}$, and moderate $\alpha N_{(i,i+3)}$, which are characteristic of an α-helix. Finally, the assignments were confirmed by a 3D CBCA(CO)NH experiment which gives additional information on the chemical shifts of $C\alpha$ and $C\beta$ of the residue preceding a backbone amide group. The resonances for residues Met-76, Lys-77 and Asp-78 could not be observed by the methods employed in this study, presumably due to the line broadening of the fast exchanging amide protons. These three residues are part of the connecting linker between the two domains of CaM, which is known to be flexible in solution (Barbato et al. 1992). The first three N-terminal residues of CaM were also missing in the spectra. Other unassigned residues include Phe-92, His-107 and Asp-118.

The secondary structural elements of Ca^{2+}-CaM complexed with the MRP peptide were deduced on the basis of NOE connectivities involving NH, $C^{\alpha}H$ and $C^{\beta}H$ protons and $C\alpha$ and $C\beta$ chemical shifts (Spera and Bax 1991). The NMR analysis revealed that the secondary structure of Ca^{2+}-CaM complexes with the MRP peptide is similar to that of Ca^{2+}-CaM in solution (Ikura et al. 1990), except for the center linker region. The central linker region of CaM, when complexed with the MRP peptide, was disrupted at residues Lys-75 to Glu-82, as evidenced by the lack of $NN_{(i,i+1)}$, $\alpha N_{(i,i+3)}$, and $\beta N_{(i,i+3)}$ connectivities (Fig. 3). The extensive broadening of the backbone amide resonances of Met-76, Lys-77 and Asp-78 suggests a greater flexibility in this region. The resulting flexible domain linker is a common characteristic of CaM when complexed with a target peptide (Ikura et al. 1991).

The MARCKS (tetra-Ser), MRP and MARCKS (tetra-Asp) peptides each induced a different magnitude of chemical shift changes in the backbone amide ^{1}H and ^{15}N resonances of Ca^{2+}-CaM, as shown in Fig. 4 for a selection of amino acid residues that spans the CaM sequence. The chemical shift changes induced by the addition of peptide reflect conformational changes in CaM upon complex formation with the peptide. In all three cases, chemical shift changes were observed for both the N- and C-terminal domains of CaM, suggesting that both CaM domains are involved in the interaction with the peptides. Interestingly, the magnitude of the changes was different from one peptide to another, and correlates with the Ca^{2+} affinity of the respective complexes (Fig. 2, Table 1). A smaller overall chemical shift change, as observed for CaM complexed with MARCKS (tetra-Asp), may reflect a lower affinity of this complex, which may also reduce the Ca^{2+} binding affinity of CaM. The overall profile of chemical shift changes (Fig. 4) was rather similar between Ca^{2+}-CaM-MRP and Ca^{2+}-CaM-MARCKS (tetra-Ser) complexes.

Recent CD measurements (Coburn et al. 1993) suggest that the MARCKS peptides are unstructured in solution. The skMLCK and smMLCK peptides have also been shown to have a random-coil structure in the uncomplexed

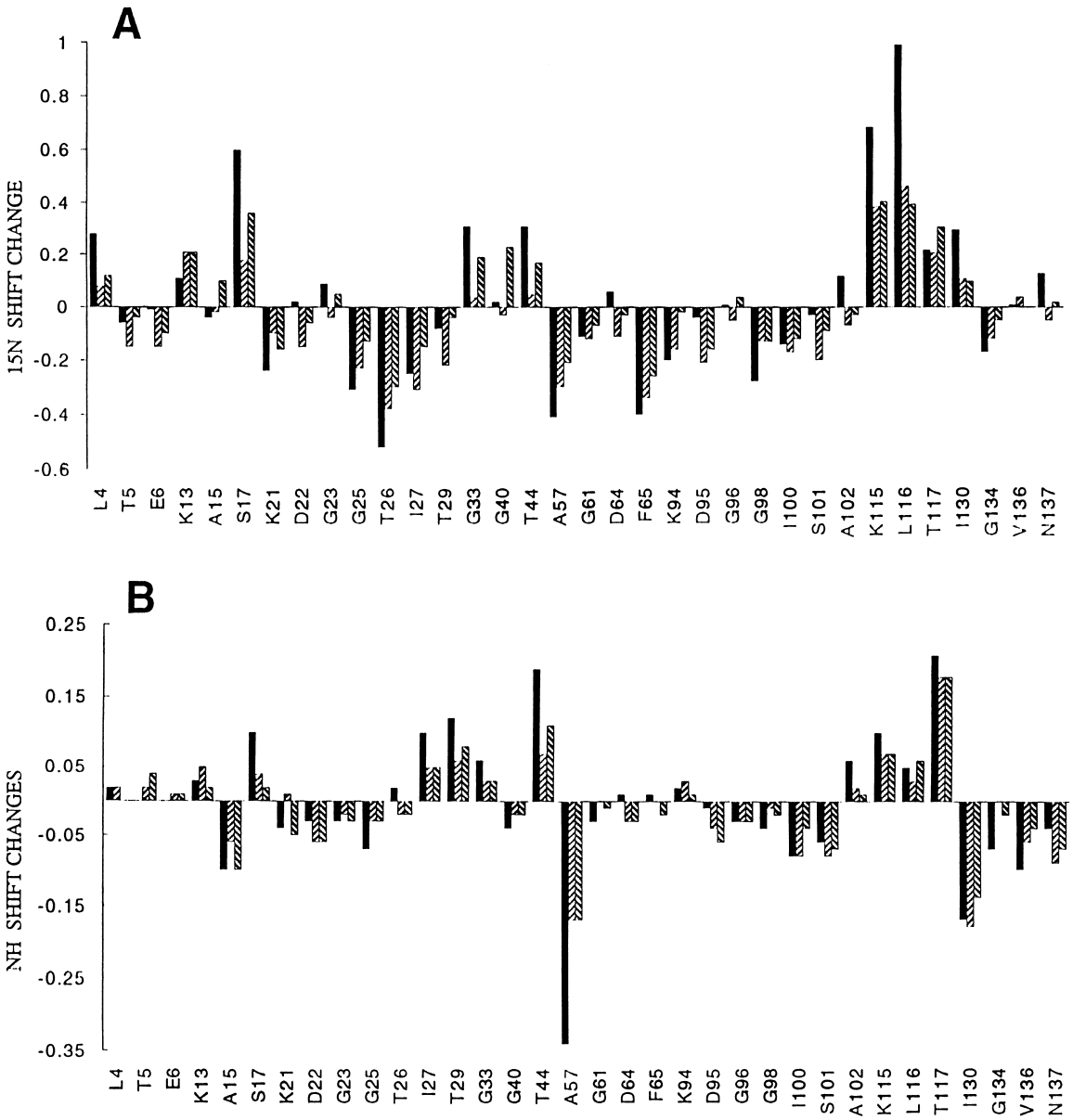


Fig. 4A, B Comparison of NMR chemical shift changes induced in Ca^{2+}-CaM upon binding of MARCKS (tetra-Ser) (■), MRP (▨) and MARCKS (tetra-Asp) peptides (▧). Chemical shift changes are shown for the ^{15}N (**A**) and NH (**B**) backbone amide resonances for a selection of amino acid residues that spans the CaM sequence

state in aqueous solution (Ikura and Bax 1992; Roth et al. 1992). However, when these MLCK peptides bind to Ca^{2+}-CaM, the peptides adopt an α-helical conformation as evidenced by CD and NMR experiments (Klevit et al. 1985; Cox et al. 1985; Giedroc et al. 1983; Erickson 1987; Ikura and Bax 1992; Roth et al. 1992; Zhang and Vogel 1994). Our CD data of CaM-MARCKS peptide complexes (Fig. 5), however, shows a decrease of the band height at 222 nm, indicating a reduction in the mean α-helical content per residue. The changes are rather subtle compared to the large increase in the band height observed for the CaM-smMLCK complex (Klevit et al. 1985). These CD results suggest that the MARCKS and MRP peptides in the complex with CaM do not assume an α-helical conformation.

^{15}N-filtered ^{1}H-^{1}H NOESY spectra (Ikura and Bax 1992) of an unlabeled MRP peptide bound to uniformly ^{15}N-labeled Ca^{2+}-CaM (Fig. 6) yielded no backbone NH/NH NOE peaks, consistent with the MRP peptide not forming an α-helical conformation in the CaM complex. Furthermore, $^{15}N/^{13}C$-filtered COSY spectra recorded with an unlabeled MRP peptide bound to $^{15}N/^{13}C$-labeled CaM (Fig. 6) show at least 12 well resolved NH/$C^{\alpha}H$ cross peaks with vicinal coupling constants of $^{3}J_{NH\alpha}$ in the range of 7–12 Hz. The relatively large coupling constants are in agreement with the non-helical conformation of the MRP peptide (Wüthrich 1986). Unfortunately, no sequence-specific assignments of the backbone amide protons could be obtained due to the high content of lysine residues in the sequence (12 out of 24 residues) which caused severe

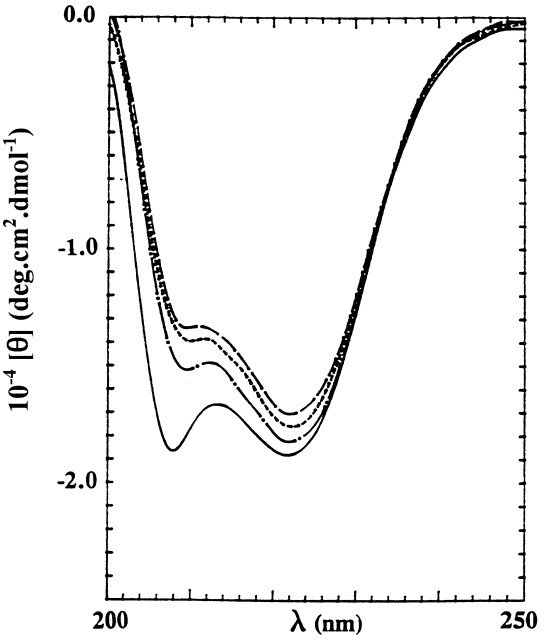
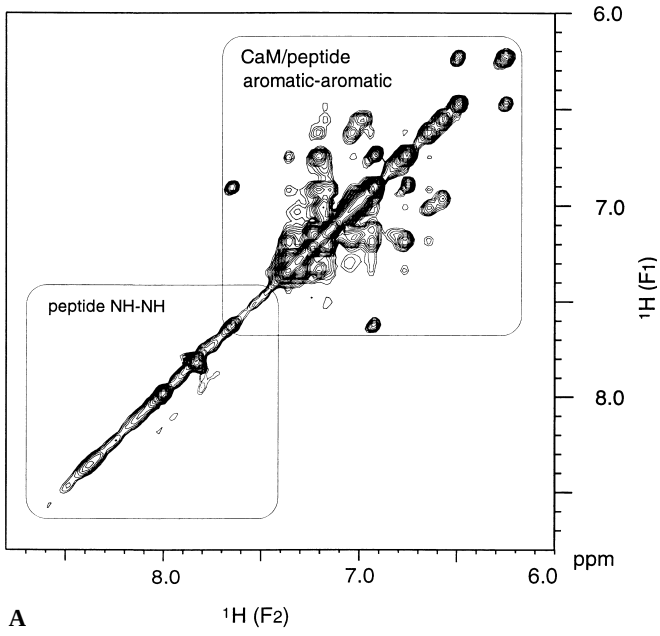


Fig. 5 CD spectra of Ca^{2+}-CaM (——) and Ca^{2+}-CaM complexed with the MARCKS (tetra-Ser) (–·–·–·–), MARCKS (tetra-Asp) (· · · · ·)and MRP (– – – –) peptides. The spectra were recorded as described under Materials and methods



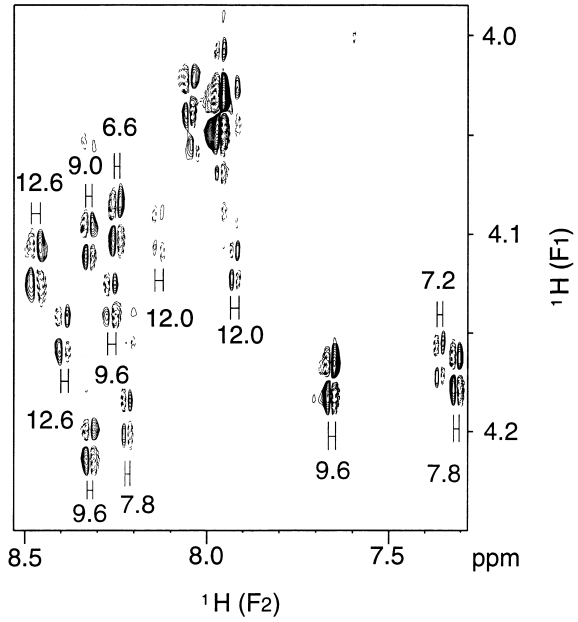


Fig. 6 **A** ^{15}N-filtered NOESY spectrum of an unlabeled MRP peptide bound to uniformly ^{15}N-labeled Ca^{2+}-CaM, showing the absence of through-space NH-NH connectivities in the peptide. **B** ^{13}C/^{15}N-filtered COSY spectrum of an unlabeled MRP peptide bound to uniformly ^{13}C/^{15}N-labeled Ca^{2+}-CaM. $^3J_{NH\alpha}$ coupling constants are shown for resolved peaks

peak overlaps in ^{13}C/^{15}N-filtered COSY and NOESY spectra. Nevertheless, these NMR data further support that the MRP peptide is non-helical in the complex with CaM.

Discussion

The results presented here are summarized as follows: (1) Both MARCKS (tetra-Ser) and MRP peptides increase the Ca^{2+} binding affinity of CaM, with enhanced positive cooperativity, whereas MARCKS (tetra-Asp) shows little effect on Ca^{2+} binding by CaM; (2) The cooperative Ca^{2+} binding behavior and the 3D NMR analysis suggest that both the N-terminal and C-terminal domains of CaM are involved in binding of the MRP and MARCKS (tetra-Ser) peptides; (3) The central helical linker of CaM in the Ca^{2+}-CaM-MRP peptide complex is disrupted between residues 75 and 82; (4) The NMR and CD data strongly suggest that the MRP peptide in the CaM complex is non-helical.

The MARCKS and MRP peptides used in this study serve as models of the CaM binding and phosphorylation domains of the intact proteins. For example, both peptides are phosphorylated by PKC with similar affinities and positive cooperativities to the intact proteins (Graff et al. 1991; Verghese et al. 1994). Both peptides display CaM binding affinities similar to those of the intact protein, and both affinities are strikingly decreased by PKC-dependent phosphorylation or replacement of the phosphorylatable serine residues by aspartate residues (Hinrichsen and Blackshear 1993; Verghese et al. 1994; Graff et al. 1989; McIlroy et al. 1991). PKC-dependent CaM binding to the peptides has also been demonstrated in intact *Paramecia*, in which the microinjected peptides affected CaM-dependent behaviors in a PKC-regulated manner (Hinrichsen and Blackshear 1993). Finally, the association of these peptides with lipid membranes has been demonstrated (Kim et al. 1994).

Our NMR experiments revealed that all three peptides studied here induce significant chemical shift changes for many residues in both the N- and C-terminal domains of CaM (Fig. 4), suggesting that both domains are involved in binding the MARCKS peptides. The magnitude of chemical shift changes varies from large (MARCKS (tetra-Ser)), medium (MRP), to small (MARCKS (tetra-Asp)). This correlates with the strength of peptide binding of CaM (K_d values around 3 nM for MARCKS (tetra-Ser) and MRP, and about ten-fold as large for MARCKS (tetra-Asp)), and with the magnitude of the Ca^{2+} binding contants of CaM complexed with the MARCKS/MRP peptides (Table 1). The peptide-induced increase in Ca^{2+} binding may be manifested by the enhanced positive allosteric interaction between Ca^{2+} binding sites in the presence of the target peptide. In other words, binding of Ca^{2+} and peptide both contribute to the stabilization of the Ca^{2+}-bound conformation of CaM.

The Ca^{2+} dissociation constants of CaM complexed with the MARCKS/MRP peptides are higher than the constant obtained previously for CaM complexed with the skMLCK peptide (Porumb et al. 1994). This suggests that the MARCKS/MRP peptides bind to CaM in a manner different from that in the CaM-skMLCK complex (Ikura et al. 1992). Our CD and NMR results indicate that the MARCKS peptides assume non-helical conformations when bound to Ca^{2+}-CaM. This contrasts with MLCK and CaM-dependent protein kinase II (CaMKII) peptides which form an α-helix when complexed with Ca^{2+}-CaM (Ikura et al. 1992; Meador et al. 1992 and 1993). The NMR data suggest that at least eight amino acid residues having $^3J_{NH\alpha}$ coupling constants larger than 9 Hz (Fig. 6) are in an extended conformation. The glycine residue found in a conserved position in the MARCKS peptides (Fig. 1) might be partially responsible for the inability of these peptides to adopt an α-helical conformation.

Although the Ca^{2+} binding constants differ from one complex to another, the Hill coefficient values of Ca^{2+} binding to CaM complexed with MARCKS (tetra-Ser) and with MRP are similar to the value of CaM complexed with the skMLCK peptide and with the individual Hill coefficient values of the domains of peptide-free CaM (Table 1). The cooperativity in Ca^{2+} binding observed for CaM complexed with the MARCKS (tetra-Ser) and MRP peptides means that the two Ca^{2+} binding domains of CaM bind Ca^{2+} almost simultaneously. The process may be understood as arising from the concomitant activation of the Ca^{2+} binding domains, upon binding of the peptide at the two terminal domains of CaM. The above results suggest that the peptides bind to the two domains of CaM simultaneously, even though the bound peptide assumes a non-helical conformation. It has been reported that the CaM binding domains of phosphorylase kinase (Dasgupta et al. 1989) and Ca^{2+}-ATPase (Falchetto et al. 1992; Kataoka et al. 1991) may bind Ca^{2+}-CaM in extended conformations. Together, these results indicate that CaM may recognize diverse binding domain conformations from extended to helical ones.

In the case of the MLCK target peptides, two hydrophobic residues situated 14 residues apart (Trp-4 nd Phe-17 in the skMLCK peptide, and Trp-5 and Leu-18 in the smMLCK peptide) bind to the hydrophobic pockets in the C-terminal and N-terminal domains of CaM, respectively (Ikura et al. 1992; Meador et al. 1992; Crivici and Ikura 1995). These hydrophobic residues serve to anchor the respective peptide to the two halves of the CaM molecule. The same mode of interaction is retained in the case of the complex of CaM with the CAMKII peptide, except that the hydrophobic anchors are provided by two leucine residues of the CaMKII peptide that are separated by 10 residues (Meador et al. 1993). The identification of the hydrophobic amino acid residues of the MARCKS/MRP peptides which bind the N- and C-terminal globular domains of the CaM can be only speculative. Such anchoring points could be Phe-6 (or 8) and Phe-19 of the MRP peptide and Phe-7 (or 9) and Phe-20 of the MARCKS peptide, based on the sequence alignment shown in Fig. 1. Although the spacing between the two phenylalanine residues (12 residues) is comparable to that of the MLCK peptide (Ikura et al. 1992; Meador et al. 1992), the non-helical conformation suggests that such anchoring points could occur almost anywhere in the sequence. Another characteristic in the CaM binding sequence is a proline residue at position 11 in MRP, corresponding to the second serine residue in MARCKS. However, our Ca^{2+} binding and spectroscopic data of the MARCKS (tetra-Ser) peptide are similar to those of the MRP peptide. This similarity between the MARCKS and MRP peptides suggests that the proline residue in the MRP peptide has little impact on binding to Ca^{2+}-CaM. This is consistent with the hypothesis that the MARCKS peptides are non-helical in the complex with CaM.

The flexible linker region (residues 78–81) in Ca^{2+}-CaM (Ikura et al. 1991; Barbato et al. 1992) is crucial for the ability of CaM to accommodate various target sequences with different surface characters. Table 2 summarizes the extent of disruption of the α-helical structure of this linker in a series of CaM-peptide complexes studied by NMR or X-ray diffraction, compared to peptide-free CaM. In the Ca^{2+}-CaM-MRP peptide complex, the flexible linker appears to consist of residues 75–82 (Fig. 3), which is comparable in length to that observed for the other Ca^{2+}-CaM-peptide complexes (Table 2). The flexibility of the linker region allows CaM to change the relative orien-

Table 2 Residues in the central helix of CaM that serve as a flexible linker in various Ca^{2+}-CaM-peptide complexes and peptide-free Ca^{2+}-CaM

CaM complex	Method	CaM linker	Reference
skMLCK	NMR	74–82	Ikura et al. 1992
smMLCK	X-ray	73–77	Meador et al. 1992 and 1993
CaMKII	X-ray	73–83	Meador et al. 1993
MRP	NMR	75–82	Present study
CaM alone	NMR	78–81	Ikura et al. 1991; Barbato et al. 1992
CaM alone	X-ray	–[a]	Babu et al. 1988

[a] Note that residues 78–81 have high temperature factors

tation of two globular domains each having a hydrophobic target binding site, such that it can accommodate various target polypeptides whose conformation can be α-helical but also extended. Finally, the similarity in the structural properties between the MARCKS and MRP peptides described here, together with the similarity in biochemical and functional properties between the intact MARCKS and MRP proteins (Verghese et al. 1994), suggest that the two proteins may serve their physiological roles by interacting with Ca^{2+}-CaM in a similar manner.

Acknowledgements We thank Charles Deber for kind use of a CD spectrometer and Rieko Ishima for discussion and help in isotope-filtered NMR experiments. We thank Lewis Kay for providing pulse sequences, Frank Delaglio for providing nmrPipe and nmrDraw, and Dan Garrett for supplying the programs Pipp, Capp, and Stapp. This work was supported by grants from Medical Research of Council of Canada (M.I.). M.I. is a recipient of a Medical Research Council of Canada Scholarship and P.J.B. is an Investigator of the Howard Hughes Medical Institute.

References

Aderem A (1992a) The MARCKS brothers: a family of protein kinase C substrates. Cell 71:713–716

Aderem A (1992b) Signal transduction and the actin cytoskeleton: the roles of MARCKS and profilin. Trends Biochem Sci 17:438–443

Ames JB, Tanaka T, Stryer L, Ikura M (1994) Secondary structure of myristoylated recoverin determined by three-dimensional heteronuclear NMR: implications for the calcium-myristoyl switch. Biochemistry 33:10743–10753

Babu YS, Bugg CE, Cook WJ (1988) Structure of calmodulin refined at 2.2 Å resolution. J Mol Biol 204:191–204

Barbato G, Ikura M, Kay LE, Pastor RW, Bax A (1992) Backbone dynamics of calmodulin studied by ^{15}N relaxation using inverse detected two-dimensional NMR spectroscopy: the central helix is flexible. Biochemistry 31:5269–5278

Blackshear PJ, Verghese GM, Johnson JD, Haupt DM, Stumpo DJ (1992) Characteristics of the F52 protein, a MARCKS homologue. J Biol Chem 267:13540–13546

Blackshear PJ (1993) The MARCKS family of cellular protein kinase C substrates. J Biol Chem 268:1501–1504

Cohen P, Klee CB (eds) (1988) Calmodulin. Elsevier, Amsterdam

Coburn C, Eisenberg J, Eisenberg M, McLaughlin S, Runnels L (1993) Calculations of electrostatic potentials adjacent to membranes and peptides that mimic the calmodulin-binding domains of neuromodulin and the MARCKS protein. Biophys J 64:A60

Cox JA, Comte M, Mamar-Bachi A, Milos M, Schaer JJ (1988) Cation binding to calmodulin and relation to function. In: Gerday C, Bolis L, Giles R (eds) Calcium and binding proteins. Springer, Berlin, pp 141–162

Cox JA, Comte M, Fitton JE, Degrado WF (1985) The interaction of calmodulin with amphiphilic peptides. J Biol Chem 260:2527–2534

Crivici A, Ikura M (1995) Molecular and structural basis of target recognition by calmodulin. Annu Rev Biophys Biomol Struct 24:85–116

Dahlquist FW (1979) The measuring of Scatchard and Hill plots. Methods Enzymol 48:270–299

Dasgupta M, Honeycutt T, Blumenthal DK (1989) The γ-subunit of skeletal muscle phosphorylase kinase contains two noncontiguous domains that act in concert to bind calmodulin. J Biol Chem 264:17156–17163

Davis TN (1992) What is new with calcium? Cell 71:557–564

DeGrado WF (1988) Design of peptides and proteins. Adv Protein Chem 39:51–125

Erickson-Viitanen S, Degrado WF (1987) Recognition and characterization of calmodulin-binding sequences in peptides and proteins. Methods Enzymol 139:455–468

Falchetto R, Vorherr T, Carafoli E (1992) The calmodulin-binding site of the plasma membrane Ca^{2+} pump interacts with the transduction domain of the enzyme. Protein Sci 1:1613–1621

Forsén S, Vogel HJ, Drakenberg T (1986) Biophysical studies of calmodulin. In: Cheung WJ (ed) Calcium and cell function, vol 6. Academic Press, New York, pp 113–157

Giedroc DP, Ling N, Puett D (1983) Identification of beta-endorphin residues 14–25 as a region involved in the inhibition of calmodulin-stimulated phosphodiesterase activity. Biochemistry 22:5584–5591

Graff JM, Young TN, Johnson JD, Blackshear PJ (1989) Phosphorylation-regulated calmodulin binding to a prominent cellular substrate for protein kinase C. J Biol Chem 264:21818–21823

Graff JM, Rajan RR, Randal RR, Nairn AC, Blackshear PJ (1991) Protein kinase C substrate and inhibitor characteristics of peptides derived from the myristoylated alanine-rich C kinase substrate (MARCKS) protein phosphorylation site domain. J Biol Chem 266:14390–14398

Hinrichsen RD, Blackshear PJ (1993) Regulation of peptide-calmodulin complexes by protein kinase C in vivo. Proc Natl Acad Sci USA 90:1585–1589

Ikura M, Kay LE, Bax A (1990) A novel approach for sequential assignment of ^{1}H, ^{13}C and ^{15}N spectra of larger proteins: heteronuclear triple resonance 3D NMR spectroscopy. Application to calmodulin. Biochemistry 29:4659–4667

Ikura M, Kay LE, Krinks M, Bax A (1991) Triple-resonance multidimensional NMR study of calmodulin complexed with the binding domain of skeletal muscle myosin light-chain kinase: indication of a conformational change in the central helix. Biochemistry 30:5498–5504

Ikura M, Bax A (1992) Isotope filtered 2D NMR of a protein-peptide complex: study of the myosin light chain kinase fragment bound to calmodulin. J Am Chem Soc 114:2433–2440

Ikura M, Clore GM, Gronenborn AM, Zhu G, Klee CB, Bax A (1992) Solution structure of a calmodulin-target peptide complex by multi-dimensional NMR. Science 256:632–638

Kay LE, Keifer P, Saarinen T (1992) Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. J Am Chem Soc 114:10663–10665

Kataoka M, Head JF, Vorherr T, Krebs J, Carafoli E (1991) Small-angle x-ray scattering study of calmodulin bound to two peptides corresponding to parts of the calmodulin-binding domain of the plasma membrane Ca^{2+} pump. Biochemistry 30:6247–6251

Kim J, Blackshear PJ, Johnson JD, McLaughlin S (1994) Phosphorylation reverses the membrane association of peptides that correspond to the basic domains of MARCKS and neuromodulin. Biophys J 67:227–237

Klee CB (1988) Interaction of calmodulin with Ca^{2+} and target proteins. In: Cohen P, Klee CB (eds) Calmodulin. Elsevier, Amsterdam, pp 35–56

Klevit RE, Blumenthal DK, Wemmer DE, Krebs EG (1985) Interaction of calmodulin with a calcium-binding peptide from myosin light chain kinase: major spectral changes in both occur as a result of complex formation. Biochemistry 24:8152–8157

Leatherbarrow RJ (1987) Enzfitter. Elsevier Biosoft, Cambridge

Lee W, Revington MJ, Arrowsmith C, Kay LE (1994) A pulsed field gradient isotope-filtered 3D ^{13}C HMQC-NOESY experiment for extracting intramolecular NOE contacts in molecular complexes. FEBS Lett 350:87–90

Li J, Aderem A (1992) MacMARCKS, a novel member of the MARCKS family of protein kinase C substrates. Cell 70:791–801

Maune JF, Klee CB, Beckingham K (1992) Ca^{2+} binding and conformational change in two series of point mutations to the individual Ca^{2+} binding sites of calmodulin. J Biol Chem 267:5286–5295

McIlroy BK, Walters JD, Blackshear PJ, Johnson JD (1991) Phosphorylation-dependent binding of a synthetic MARCKS peptide to calmodulin. J Biol Chem 266:4959–4964

Meador WE, Means AR, Quiocho FA (1992) Target enzyme recognition by calmodulin: 2.4 Å structure of a calmodulin-peptide complex. Science 257:1251–1255

Meador WE, Means AR, Quiocho FA (1993) Modulation of calmodulin plasticity in molecular recognition on the basis of X-ray structure. Science 262:1718–1721

Minowa O, Yagi K (1984) Calcium binding to tryptic fragments of calmodulin. J Biochem 96:1175–1182

O'Neil KT, Degrado WF (1990) How calmodulin binds its targets: sequence independent recognition of amphiphilic α-helices. Trends Biochem Sci 15:50–64

Porumb T, Yau P, Harvey TS, Ikura M (1994) A calmodulin-target peptide hybrid molecule with unique calcium-binding properties. Protein Engng 7:109–115

Porumb T (1994) Determination of calcium-binding constants by flow dialysis. Anal Biochem 220:227–237

Roth SM, Schneider DM, Strobel LA, VanBerkum MFA, Means AR, Wand AJ (1991) Structure of the smooth muscle myosin light-chain kinase calmodulin-binding domain peptide bound to calmodulin. Biochemistry 30:10078–10084

Spera S, Bax A (1991) Empirical correlation between protein backbone conformation and Cα and Cβ 13C NMR chemical shifts. J Am Chem Soc 113:5490–5492

Starovasnik MA, Davis TN, Klevit RE (1993) Similarities and differences between yeast and vertebrate calmodulin: an examination of the calcium-binding and structural properties of calmodulin from the yeast Saccharomyces cerevisiae. Biochemistry 32:3261–3270

Stumpo DJ, Graff JM, Albert KA, Greengard P, Blackshear PJ (1989) Molecular cloning, characterization, and expression of a cDNA encoding the "80- to 87-kDa" myristoylated alanine-rich C kinase substrate: a major cellular substrate for protein kinase C. Proc Natl Acad Sci USA 86:4012–4016

Takio K, Blumenthal DK, Edelman AM, Walsh KA, Krebs EG, Titani K (1985) Amino acid sequence of an active fragment of rabbit skeletal muscle myosin light chain kinase. Biochemistry 24:6028–6037

Verghese GM, Johnson JD, Vasulka C, Haupt DM, Stumpo DJ, Blackshear PJ (1994) Protein kinase C-mediated phosphorylation and calmodulin binding of recombinant myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS-related protein. J Biol Chem 269:1–7

Wüthrich K (1986) NMR of proteins and nucleic acids. Wiley, New York, NY

Yazawa M, Sakuma M, Yagi K (1984) Calmodulins from muscles of marine invertebrates, scallop and sea anemone. J Biochem 87:1313–1320

Zhang M, Vogel HJ (1994) Characterization of the calmodulin-binding domain of rat cerebellar nitric oxide synthase. J Biol Chem 269:981–985