REVIEW / SYNTHÈSE

Molecular mechanisms of calmodulin's functional versatility

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Abstract: Calmodulin (CaM) is a primary Ca^{2+}-binding protein found in all eukaryotic cells. It couples the intracellular Ca^{2+} signal to many essential cellular events by binding and regulating the activities of more than 40 different proteins and enzymes in a Ca^{2+}-dependent manner. CaM contains two structurally similar domains connected by a flexible central linker. Each domain of the protein binds two Ca^{2+} ions with positive cooperativity. The binding of Ca^{2+} transforms the protein into its active form through a reorientation of the existing helices of the protein. The two helices in each helix–loop–helix Ca^{2+}-binding motif are almost antiparallel in Ca^{2+}-free CaM. The binding of Ca^{2+} induces concerted helical pair movements and changes the two helices in each Ca^{2+} binding motif to a nearly perpendicular orientation. These concerted helix pair movements are accompanied by dramatic changes on the molecular surface of the protein. Rather than exhibiting a flat, hydrophilic molecular surface as seen in Ca^{2+}-free CaM, the Ca^{2+}-saturated form of the protein contains a Met-rich, cavity-containing hydrophobic surface in each domain. These hydrophobic surfaces are largely responsible for the binding of CaM to its targets. The unique flexibility and high polarizability of the Met residues located at the entrance of each hydrophobic pocket together with other hydrophobic amino acid residues create adjustable, sticky interaction surface areas that can accommodate CaM's targets, which have various sizes and shapes. Therefore, CaM is able to bind to a large array of targets without obvious sequence homology. Upon binding to its target peptides, the unwinding of the central linker allows the two domains of the protein to engulf the hydrophobic face of target peptides of differing lengths. The binding of Ca^{2+} reduces the backbone flexibility of CaM. Formation of complexes with its target peptides further decreases the backbone motion of CaM.

Key words: calmodulin, NMR, structure and dynamics, peptide targets, ligand specificity.

Résumé : La calmoduline (CaM) est une protéine liant le Ca^{2+} importante qui se trouve dans toutes les cellules eucaryotes. Elle couple le signal intracellulaire du Ca^{2+} à plusieurs processus cellulaires essentiels en se liant d'une façon Ca^{2+} dépendante à plus de 40 protéines et enzymes différentes et en réglant leurs activités. La CaM comporte deux domaines ayant des structures semblables et qui sont reliés par un bras central flexible. Chaque domaine de la protéine lie deux ions Ca^{2+} grâce à une coopérativité positive. La liaison du Ca^{2+} transforme la protéine en sa forme active en entraînant une réorientation des hélices de la protéine. Les deux hélices de chacun des motifs hélice–boucle–hélice liant le Ca^{2+} sont presque antiparallèles dans la CaM non liée au Ca^{2+}. La liaison du Ca^{2+} à la CaM induit des mouvements concertés de la paire d'hélices et entraîne un changement d'orientation des deux hélices de chacun des motifs liant le Ca^{2+}, qui deviennent ainsi presque perpendiculaires. Ces mouvements concertés de la paire d'hélices sont accompagnés de changements majeurs à la surface de la protéine. Au lieu d'être plate et hydrophile comme dans la CaM non liée au Ca^{2+}, la surface de chaque domaine de la CaM saturée de Ca^{2+} est hydrophobe et a une cavité riche en méthionine. La CaM se lie à ses cibles principalement grâce à ces surfaces hydrophobes. La flexibilité exceptionnelle et la forte polarisation des résidus de méthionine localisés à l'entrée de chacune des cavités hydrophobes et d'autres résidus d'acides aminés hydrophobes créent des surfaces d'interaction adaptables et adhésives qui peuvent s'ajuster aux cibles de la CaM, de formes et de dimensions variées. Par

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Abbreviations: CaM, calmodulin; CaMKI, CaM-dependent protein kinase I; CaMKII, CaM-dependent protein kinase II; CaD, caldesmon; CD, circular dichroism; ELC, essential light chain; FTIR, Fourier transformed infrared; MLCK, myosin light chain kinase; M13 peptide, CaM-binding domain of skeletal muscle MLCK; NOE, nuclear Overhauser effect; NOS, nitric oxide synthase; PDE, phosphodiesterase; Phk, phosphorylase kinase; SeMet, selenomethionine; TrNOE; transferred nuclear Overhauser enhancement.

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conséquent, la CaM peut se lier à un large éventail de cibles sans homologie de séquence évidente. Lors de la liaison de la CaM aux peptides cibles, le déploiement du bras central permet aux deux domaines de la protéine d'engouffrer la face hydrophobe de peptides cibles de différentes longueurs. La liaison du Ca^{2+} réduit la flexibilité du squelette de la CaM. La formation du complexe avec les peptides cibles diminue encore plus le mouvement du squelette de la CaM.

Mots clés : calmoduline, résonance magnétique nucléaire, RMN, structure, dynamique, cibles peptidiques, spécificité de ligand.

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Introduction

The cellular free Ca^{2+} concentration of living cells is tightly controlled within the range of 10^{-7}~10^{-5} M via intricate Ca^{2+} influx and efflux mechanisms. Upon external stimulation, the intracellular Ca^{2+} concentration transiently increases to ~10^{-5} M, resulting in the calcification of an array of Ca^{2+}-receptor proteins. The binding of Ca^{2+} results in conformational changes of these Ca^{2+}-binding proteins, thus enabling these proteins to interact with their respective partner proteins and (or) enzymes. The formation of complexes between Ca^{2+}-binding proteins and their targets induces further conformational changes in both components of the complexes, thereby allowing functional regulation of the targets. Therefore, Ca^{2+}-binding proteins play pivotal roles in Ca^{2+}-mediated signal transduction pathways.

Calmodulin (CaM) is a primary Ca^{2+}-receptor protein. It is a small (148 amino acid residues), highly conserved protein molecule found in all eukaryotes. The protein is able to bind to at least 40 different target proteins and enzymes including various protein kinases and phosphatases, receptors, ion-channel proteins, phosphodiesterases, and nitric oxide synthases. These CaM targets play essential roles in a wide variety of cellular events such as cytoskeletal dynamics, metabolism, cell proliferation, and development (for recent reviews, see Vogel 1994; James et al. 1995; Crivici and Ikura 1995). CaM consists of two similar domains, each containing two Ca^{2+}-binding sites. Each metal-ion binding site is made up of a so-called EF-hand helix–loop–helix motif. The C-terminal domain of CaM cooperatively binds to two Ca^{2+} ions with a K_d of ~10^{-6} M, and the N-terminal pair of EF-hands also cooperatively binds to Ca^{2+} ions, albeit with an approximately 10-fold lower affinity (Ikura et al. 1983; Andersson et al. 1983; Dalgarno et al. 1984; Thulin et al. 1984). The Ca^{2+}-binding affinity of CaM falls exactly in the range of the intracellular Ca^{2+} concentration fluctuation, ensuring that the protein is capable of responding to cellular Ca^{2+} signaling. In addition, the kinetic parameters of Ca^{2+} binding (k_{on} ~10^8 s^{-1} and k_{off} ~10^3 s^{-1}, a diffusion controlled on-rate and a millisecond off-rate) allow the protein to respond in a timely fashion to transient cellular Ca^{2+} concentration fluctuations. Therefore, CaM is ideally suited as a cellular Ca^{2+} signal trigger both thermodynamically and kinetically. The binding of Ca^{2+} to CaM induces large conformational changes in both domains of the protein. The Ca^{2+}-bound form of CaM is competent in binding with high affinity (with a K_d of ~10^{-8} to 10^{-11} M) to its targets, and thereby activating these proteins and enzymes.

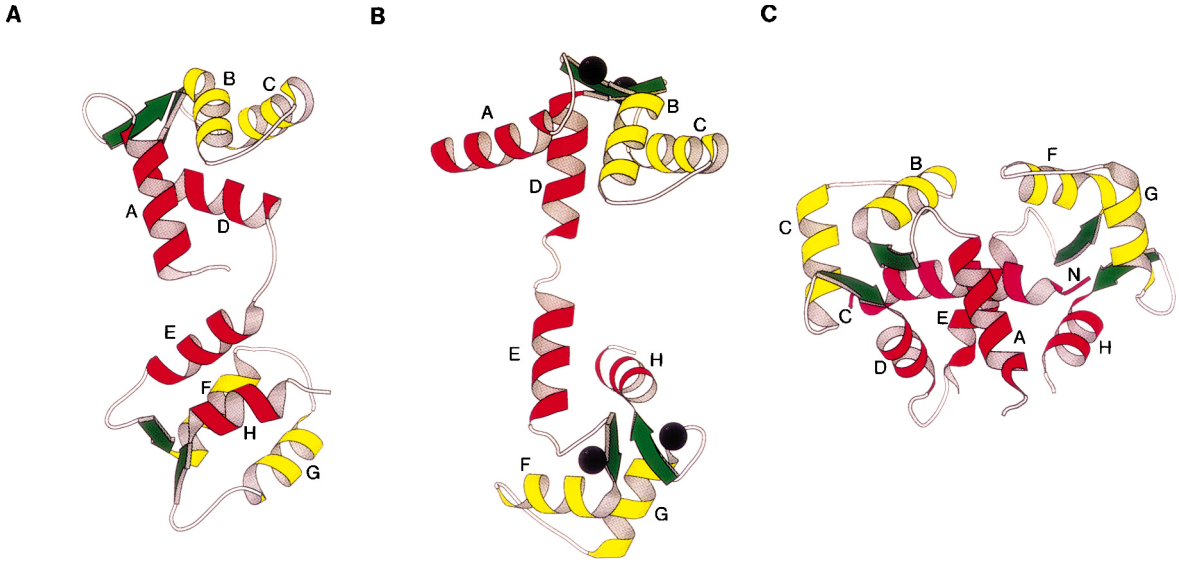
A number of excellent reviews have been published in recent years describing various aspects of the structural and functional properties of CaM (Means et al. 1991; Vogel 1994; Crivici and Ikura 1995; James et al. 1995; Vogel and Zhang 1995; Ikura 1996). In this review, we focus on the Ca^{2+}-activation mechanism of CaM by comparing the structure and dynamics of apo-CaM and Ca^{2+}-CaM. Recent progress in the area of the interactions between CaM and its target peptides will also be discussed. Finally, we try to explain the structural basis of CaM's low sequence specificity and high binding affinity towards its various targets.

Ca^{2+}-induced conformational changes

The crystal structure of Ca^{2+}-saturated CaM (Babu et al. 1988) has shown that the protein has a dumbbell shape comprising an N- and a C-terminal globular domain, which are connected by a long, solvent-exposed α-helix, the so-called central helix (Fig. 1B). The two helices in each of the four helix–loop–helix (the so-called EF-hand) Ca^{2+}-binding sites are almost perpendicular to each other. Each Ca^{2+}-binding site is comprised of a highly conserved, continuous stretch of 12 residues. The residues at positions 1, 3, 5, 7, and 9 of this 12-residue loop each provide one ligand, and the side chain of the 12th residue supplies two oxygen ligands to each Ca^{2+} ion. Hence, Ca^{2+} forms a pseudo-bipyramidal coordination sphere in Ca^{2+}-saturated CaM (Babu et al. 1988). Residues 7–9 of each Ca^{2+}-binding loop form a mini β-strand, and the two β-strands in each domain form an antiparallel sheet structure (Fig. 1B). Other than forming the antiparallel β-sheet, the helices of two EF-hands pack with each other to form a compact, globular domain structure. In particular, extensive contacts between the first and fourth (helices A–D for the N-terminal domain and E–H for the C-terminal domain) and between the second and third helices (helices B–C and F–G) of each domain are observed (Fig. 1).

The recently determined solution structure of Ca^{2+}-free CaM (Kuboniwa et al. 1995; Zhang et al. 1995*a*) has shown that apo-CaM has a secondary structure identical to that of Ca^{2+}-saturated CaM (Figs. 1A and 1B). In contrast to the perpendicular orientation seen in Ca^{2+}-CaM, the two helices of each helix–loop–helix unit in apo-CaM run almost antiparallel to each other. The two EF-hands in each domain of the apo-protein also fold into a compact structure. Similar to what was observed in Ca^{2+}-CaM, intimate contacts between the first and fourth, and between the second and third, helices of both domains are maintained. An antiparallel β-sheet structure formed between the two Ca^{2+} binding sites of each domain was also detected in Ca^{2+}-free CaM, although the β-sheets in apo-CaM are somewhat distorted. As was determined for the Ca^{2+}-saturated CaM in solution (Barbato et al. 1992), the two domains of apo-CaM are connected by a flexible linker spanning residues 77 to 80. The binding of Ca^{2+} changes the two helices of each EF-hand from a nearly antiparallel orientation (closed conformation)

Fig. 1. Schematic diagrams of the three-dimensional structures of (A) Ca^{2+}-free CaM (Zhang et al. 1995*a*; lDMO), (B) Ca^{2+}-saturated CaM (Babu et al. 1988; 3CLN), and (C) the complex of CaM with the skMLCK peptide (Ikura et al. 1992; 2BBM). The first and fourth helix pair and the second and third helix pair in each domain of the protein are shown in red and yellow, respectively, to show the concerted movements of the helix pairs upon Ca^{2+} binding. The black spheres in B represent Ca^{2+} ions of the protein. The skMLCK peptide in the complex is shown in purple, and its N- and C-termini are labeled with N and C, respectively. The figure was prepared using MOLSCRIPT (Kraulis 1991).



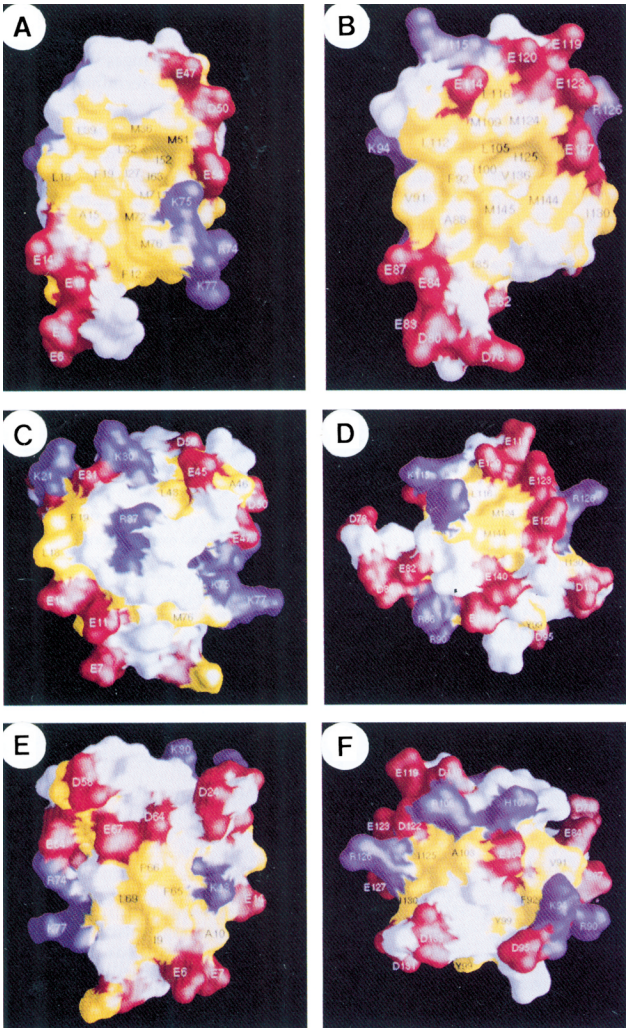
to an almost perpendicular direction (open conformation). Such Ca^{2+}-induced conformational changes are the result of concerted movements of the helix pair A–D (E–H for the C-terminal domain) with respect to the helix pair B–C (F–G for the C-terminal domain). It was proposed that such helix pairing could be the key structural basis for the Ca^{2+}-binding cooperativity observed for both domains of CaM (Zhang et al. 1995*a*). Similar concerted helix pair readjustments have also been observed in troponin C (Strynadka., and James 1991; Gagne et al. 1995; Strynadka et al. 1997; Houdusse et al. 1997), and this is likely to be a general mechanism for regulatory EF-hand Ca^{2+}-binding proteins. In contrast, structural Ca^{2+}-binding proteins and domains such as calbindin D_{9K} (Skelton et al. 1994) and the C-terminal domain of recoverin (Tanaka et al. 1995; Ames et al. 1997) do not show such large helix orientation changes upon Ca^{2+} binding.

Comparisons of the surface structures of apo-CaM and Ca^{2+}-saturated CaM have provided an answer to the question of the Ca^{2+} dependence of CaM's interaction with its targets. The crystal structures of Ca^{2+}-CaM have shown that this form of the protein contains a large, solvent-exposed hydrophobic surface in the each domain (Fig. 2). These hydrophobic surfaces have been shown to be largely responsible for the binding of CaM to its targets (Ikura et al. 1992; Meador et al. 1992, 1993). At the center of the hydrophobic surface, it contains a deep hydrophobic cavity that anchors bulky aromatic or long alkyl amino acid side chains of the CaM targets. The most striking difference with apo-CaM's surface structure is that the latter does not contain such a large, solvent-exposed, and cavity-containing hydrophobic surface (Fig. 2). Hence, CaM in the absence of Ca^{2+} is unable to bind to and activate the majority of its target enzymes. The binding of Ca^{2+} triggers conformational changes and transforms the protein from its inactive form to its active form in response to the rise in the cellular Ca^{2+} concentration.

Dynamics of the Apo-, Ca^{2+}-, and target-bound forms of CaM

Although both the Ca^{2+}-free and Ca^{2+}-saturated forms of CaM have an identical secondary structure in solution (Ikura et al. 1991; Kuboniwa et al. 1995; Zhang et al. 1995*a*), the secondary structure of Ca^{2+}-free CaM appears to be more flexible than that of the Ca^{2+}-saturated protein. Amide exchange experiments have shown that the amide protons of the amino acid residues in the secondary structural elements of apo-CaM exchange with solvent at a much faster rate than in Ca^{2+}-CaM, indicating that hydrogen bonds in apo-CaM are weaker than in Ca^{2+}-CaM (Tjandra et al. 1995; Zhang et al. 1995*a*). We have also compared the chemical shifts of the $^{13}C\alpha$ and backbone ^{15}N between Ca^{2+}-free and Ca^{2+}-saturated CaM throughout the entire amino acid sequence. Figure 3 shows the $^{13}C\alpha$ and ^{15}N chemical shift difference between Ca^{2+}-CaM and apo-CaM as a function of amino acid residue number. It is clear that Ca^{2+}-saturated CaM has uniformly larger secondary chemical shifts in the regular secondary structural regions than Ca^{2+}-free CaM, further substantiating the view that the secondary structure of Ca^{2+}-free CaM is dynamically less stable throughout the entire protein. It is well known that the addition of Ca^{2+} to apo-CaM results in significant decreases in the ellipticity values of the circular dichroism (CD) spectrum of the protein both at 208 and 222 nm, indicative of an α-helical structure of the protein (Martin and Bayley 1986). It was interpreted that binding of Ca^{2+} to apo-CaM results in an increase in the α-helicity of the protein. However, as mentioned earlier, Ca^{2+}-free and Ca^{2+}-saturated CaM have the same amount of secondary structure in solution. We suggest that the lower negative ellipticity values at 208 and 222 nm observed for apo-CaM originate from the relatively more flexible α-helices of the protein in the absence of Ca^{2+}.

Fig. 2. The molecular surface structures of Ca^{2+}-free (Zhang et al. 1995*a*; lDMO) and Ca^{2+}-saturated CaM (Babu et al. 1988; 3CLN). A and B represent the cavity-containing hydrophobic surface areas of the N- and C-terminal domains of Ca^{2+}-saturated CaM. C and E show the surface structure of the N-terminal domain of Ca^{2+}-free CaM viewed with 180° rotation. D and F are the front and back views of the C-terminal domain structure of Ca^{2+}-free CaM. In this figure, the hydrophobic residues are shown in yellow, negatively charged residues in red, positively charged residues in magenta, and polar residues in white. (Reproduced from Zhang et al. (1995*a*) with permission.)



The reduction of the flexibility of the α-helices also has a marked effect on the FTIR spectra of the protein upon addition of Ca^{2+} (Fabian et al. 1996). In general, caution should be taken in interpreting CD and Fourier transformed infrared (FTIR) spectra of proteins as both the quantity and the flexibility of the secondary structural elements of the proteins can contribute to the signal intensity of CD spectra, or the peak position of FTIR spectra.

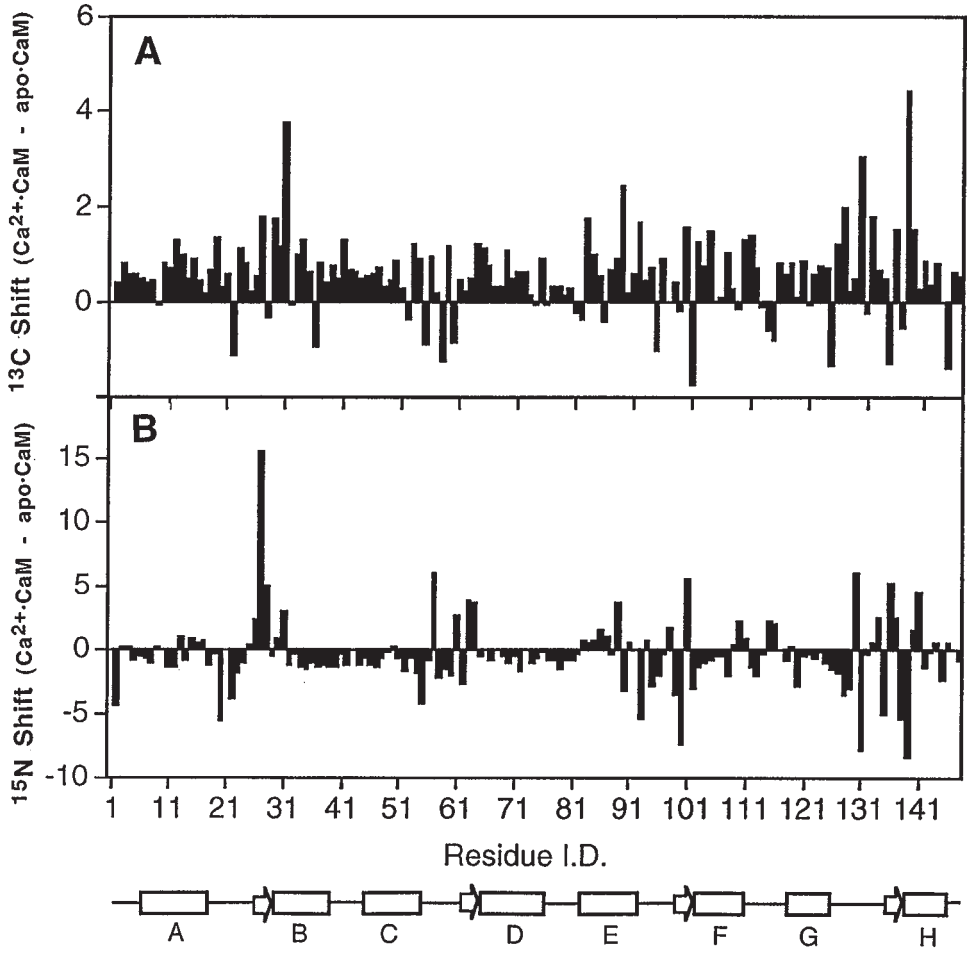
Apo-CaM is less stable than the Ca^{2+}-saturated and target-bound forms of the protein. This is particularly true for the C-terminal domain of the protein. Heat denaturation studies have shown that the C-terminal domain of Ca^{2+}-free CaM starts to denature slightly above the physiological temperature (Tsalkova and Privalov 1985), whereas Ca^{2+}-saturated CaM survives boiling in solution. It was also noted from relaxation measurements that Ca^{2+}-free CaM has more profound conformational averaging than Ca^{2+}-saturated and peptide-bound forms of the protein (Tjandra et al. 1995; M. Zhang, unpublished observations). In particular, the C-terminal domain of apo-CaM exhibits multi-conformational equilibria (Tjandra et al. 1995). The dominant conformation observed in solution by NMR (Kuboniwa et al. 1995; Zhang et al. 1995*a*) has a semi-open structure. Such a semi-open structure resembles the transition-state-like conformation in the reaction of Ca^{2+} binding to CaM that transforms CaM from a closed conformation to an open conformation. Hence the free energy (ΔG) of Ca^{2+} binding to the C-terminal domain is more negative than that of Ca^{2+} binding to the N-terminal domain, assuming that both the N-terminal and the C-terminal domains of the protein have similar free energy in the Ca^{2+}-bound state (Fig. 4). The less stable semi-open conformation of apo-CaM may provide a structural basis for the higher Ca^{2+}-binding affinity of the C-terminal domain of the protein.

The binding of Ca^{2+} to CaM significantly reduces the backbone flexibility of the protein (Barbato et al. 1992). However, the binding of Ca^{2+} creates a large flexible hydrophobic surface in each domain of CaM (see below for details), and such hydrophobic surfaces poise the protein to interact with its targets. The binding of peptide targets such as the skMLCK peptide (see below) further reduces the backbone flexibility of the protein throughout its entire sequence. The backbone amides in both N- and C-terminal domains of CaM in its complex with the skMLCK peptide have essentially uniform ^{1}H-^{15}N nuclear Overhauser effect (NOE) and order parameter (S^2) values typical of a highly ordered protein, suggesting that the complex is a rigid, isotropically tumbling molecule (M. Zhang, R. Ishima, L. Kay, and M. Ikura, unpublished observations). This notion is further underscored by the fact that the backbone relaxation parameters (T_1, T_2, and NOE) of the complex can be well fitted using model free formulism without including the conformational exchanging term R_{ex} (Lipari and Szabo 1982). Relaxation studies of the methyl dynamics have further shown that the binding of a CaM target peptide significantly reduces the side-chain flexibility of specific amino acid residues in the protein (Siivari et al. 1995; R. Ishima, N. Farrow, L. Kay, M. Zhang, and M. Ikura, unpublished observations).

Interaction of CaM with its targets

The CaM-binding domains in target proteins usually comprise a stretch of ~20 amino acid residues and they are often located at the C-terminal end or in flexible loop regions of target proteins (for reviews see O'Neil and DeGrado 1990; Crivici and Ikura 1995; Vogel and Zhang 1995; Rhoads and Friedberg 1997, Fig. 5). Thus, synthetic peptides with the same amino acid sequence as the target region are good model systems to study the interaction between CaM and target proteins. The majority of the CaM-binding domains contain positively charged residues and hydrophobic residues; many of them have the potential to form an amphiphilic α-helix. The hydrophobic residues of the target peptides will interact with the two hydrophobic surfaces ex-

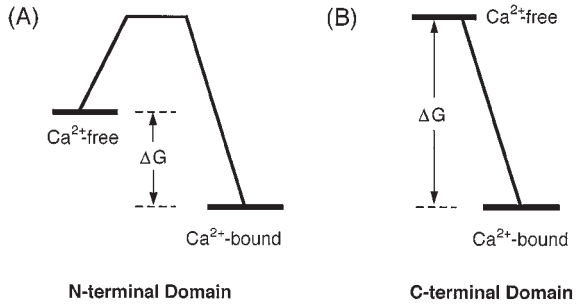
Fig. 3. Plot as a function of amino acid residue number of (A) the Cα chemical shift differences and (B) the backbone amide ^{15}N chemical shift differences of Ca^{2+}-saturated CaM to that of Ca^{2+}-free CaM. The secondary structure of the protein is schematically represented at the bottom of the figure. The open boxes show the α-helical regions, the arrows indicate the β-strands, and the straight lines indicate the coiled structures of the protein. The chemical shift values for Ca^{2+}-free CaM were taken from an earlier work (Zhang et al. 1995*a*) and the data for Ca^{2+}-saturated CaM recorded under similar conditions are courtesy of Prof. Ad Bax at the National Institute of Health.



posed in Ca^{2+}-CaM and their positively charged residues can make specific salt bridges with acidic residues in CaM (Fig. 2). The high-resolution structures of the complexes between Ca^{2+}-CaM and the CaM-binding domain peptide derived from skeletal muscle myosin light chain kinase (skMLCK) and smooth muscle myosin light chain kinase (smMLCK) were determined by NMR and X-ray crystallography, respectively (Ikura et al. 1992; Meador et al. 1992). In the Ca^{2+}-CaM-skMLCK peptide complex, each domain of Ca^{2+}-CaM retains a conformation very similar to that of Ca^{2+}-CaM (Fig. 1C). This has also been shown in solution by isotope-edited FTIR studies for several CaM – target peptide complexes (Zhang et al. 1994*a*). In contrast, the central linker region in Ca^{2+}-CaM unwinds (residues 74~82) and the N- and C-lobes of Ca^{2+}-CaM wrap around the α-helical peptide. As a result, the shape of the CaM-peptide complex is globular, as opposed to the dumbbell-shaped structure of Ca^{2+}-CaM in the crystal. The skMLCK peptide, which is unstructured in solution, forms an α-helix in the complex and its side chains make intensive interactions with the two hydrophobic patches in Ca^{2+}-CaM (Zhang et al. 1993). The skMLCK peptide binds to Ca^{2+}-CaM in an antiparallel orientation with the N-terminal of the peptide interacting predominantly with the C-lobe of Ca^{2+}-CaM (Fig. 1C). Two bulky hydrophobic residues (Trp-345 and Phe-358) separated by 12 residues anchor the peptide to the C- and N-lobes of Ca^{2+}-CaM, respectively. As a result of the formation of the complex, both hydrophobic surfaces from Ca^{2+}-CaM and the skMLCK peptide are largely buried and most of the interactions (~80%) between them involve side chain – side chain van der Waals interactions. The X-ray structure of the Ca^{2+}-CaM-smMLCK peptide complex is similar to the NMR structure of the Ca^{2+}-CaM-skMLCK peptide complex, except that the central linker region of CaM unwinds between residues 73 and 77 (Meador et al. 1992).

Subsequently, Meador et al. (1993) reported the X-ray structure of a complex between Ca^{2+}-CaM and a peptide representing the CaM-binding domain of CaM-dependent protein kinase IIa (CaMKII). The CaMKII peptide binds in a similar orientation to Ca^{2+}-CaM, that is, the N-terminal of the peptide binds to the C-lobe of Ca^{2+}-CaM. However, the two bulky hydrophobic residues (Leu-299 and Leu-308) anchoring the peptide to the protein are only separated by eight

Fig. 4. Free energy diagrams of Ca^{2+} binding to (A) the N-terminal domain and (B) the C-terminal domain of CaM. In this presentation, we assume that the N-terminal and the C-terminal domains of Ca^{2+}-saturated CaM have similar free energy. In the Ca^{2+}-free state, the C-terminal domain adopts a semi-open, transition-state-like structure. Therefore, a larger negative ΔG value (i.e., a higher Ca^{2+}-binding affinity) is observed for the Ca^{2+}-binding reaction for the C-terminal domain.



amino acid residues. To accommodate this change, the central linker region of Ca^{2+}-CaM is further unwound (residues 73–83) to enable these interactions to form. The plasticity of the central linker region was further demonstrated by X-ray structures determined for three complexes between the CaMKII peptide and Ca^{2+}-CaM mutants with the central linker region shortened by two or three residues, or replaced by the longer troponin C central linker region (Meador et al. 1995). The structures of all three mutant CaM-peptide complexes are very similar to the structure of the Ca^{2+}-CaM(wild type)-CaMKII complex (Meador et al. 1993), except that the central linker region uncoils and disposes differently for the mutant proteins. A binding pattern similar to that of the Ca^{2+}-CaM-CaMKII peptide complex has also been observed for the CaM-binding domain of caldesmon (CaD1), a protein involved in the regulation of smooth muscle contraction (Zhang and Vogel 1994*a*). Taking advantage of the relatively weak binding of the 17-residue CaD1 peptide to Ca^{2+}-CaM, these workers demonstrated that CaD1 binds to Ca^{2+}-CaM with an α-helical structure between residues Ile-655 and Val-664 using transferred nuclear Overhauser enhancement (TrNOE) NMR spectroscopy. Thus, a target peptide with a stretch of amino acid residues with two bulky hydrophobic residues separated by 8~12 residues represents one of the major binding modes for CaM binding domain peptides (Figs. 1*c*, 5).

NMR, fluorescence, and CD studies concerning the binding of peptides derived from various other target proteins to CaM, such as cerebellar nitric oxide synthase (cNOS), simian immunodeficiency virus glycoprotein, and CaM-dependent kinase I (CaMKI), also interact with both Met-rich regions on the surface of CaM and these also bind as an amphiphilic α-helix with the N-terminal region of the peptide bound to the C-terminal domain of Ca^{2+}-CaM (Yuan et al. 1995; Zhang et al. 1995*b*). Recently, we discovered a significant quenching effect on the Trp fluorescence of the bound MLCK peptide and CaMKI peptide by the unnatural amino acid selenomethionine (SeMet) in SeMet-substituted CaM (Yuan et al. 1998). The quenching properties of SeMet in Ca^{2+}-CaM can be used uniquely to determine the orientation of the MLCK peptide and CaMKI peptide bound to Ca^{2+}-CaM. These results showed that the N-terminal Trp residue in both peptides binds to the C-lobe of Ca^{2+}-CaM; this is consistent with results obtained by nitroxide spin labeled peptides and NMR spectroscopy (Zhang et al. 1995*b*).

As inferred from the structures of Ca^{2+}-CaM-MLCK peptide complexes (Ikura et al. 1992; Meador et al. 1992), an alternative orientation (N-terminal of peptide binds to the N-lobe of Ca^{2+}-CaM) is also feasible because of the pseudo two-fold symmetry between the N- and C-lobes of Ca^{2+}-CaM in the complexes. Indeed, melittin, a toxic peptide from bee venom, binds to Ca^{2+}-CaM in this orientation in the X-ray structure (Quiocho et al. 1997). We have also found that melittin binds to Ca^{2+}-CaM in a N- to N- manner using half melittin peptides and proteolytic fragments of CaM (T. Yuan and H.J. Vogel, unpublished observations). Cadmium-113 and proton NMR spectroscopy indicated that the C-half of melittin peptide binds strongly to the C-lobe of Ca^{2+}-CaM, and this interaction may initiate the binding process.

Given the multiple target proteins of CaM, alternative binding modes may be expected. Indeed, two noncontiguous segments in the regulatory domain of the catalytic subunit of phosphorylase kinase were found to bind to Ca^{2+}-CaM simultaneously (Dasgupta et al. 1989; also see below). Small angle X-ray and neutron scattering studies showed that CaM remains in an extended conformation in the complex with one of these peptides, Phkl3 (Trewhella et al. 1990). In contrast, binding of the other peptide, Phk5, induces dramatic contraction of Ca^{2+}-CaM, which is similar to the complex structures of Ca^{2+}-CaM-MLCK peptide (Ikura et al. 1992; Meador et al. 1992). Interestingly, in the presence of both Phkl3 and Phk5 peptides, Ca^{2+}-CaM remains extended (Trewhella et al. 1990). Juminaga et al. (1994) subsequently demonstrated a low α-helical content in the CD spectra upon the formation of a complex between Ca^{2+}-CaM and Phkl3 peptide. This is dramatically different from other complexes, such as Ca^{2+}-CaM-MLCK peptide or Ca^{2+}-CaM-Phk5 peptide complexes, which showed an increase in α-helical content in the CD spectra of the complexes supporting the α-helix conformation adopted by the bound peptides (Juminaga et al. 1994; Vogel and Zhang 1995). The Phkl3 peptide probably binds to Ca^{2+}-CaM with an extended hairpin-shaped conformation, as determined with the aid of a Trp-substituted Phkl3 peptide and fluorescence radiationless energy transfer experiments (Juminaga et al. 1994). Other peptides, such as a peptide from a Ras-related GTP-binding protein (Ral-A), also appear to adopt an extended conformation upon binding to Ca^{2+}-CaM (Wang et al. 1997*a*). High-resolution NMR or X-ray structures of these complexes are needed to elucidate the details of this binding mode.

In addition to the catalytic subunit of phosphorylase kinase, caldesmon has also been shown to bind to Ca^{2+}-CaM with two noncontiguous segments. Both the CaD1 and CaD2 peptide bind to Ca^{2+}-CaM with an α-helical structure, although the CaD2 peptide only forms an α-helix from Val-685 to Thr-696 (Zhang and Vogel 1994*a*; Zhou et al. 1997). Interestingly, these two peptides can interact with Ca^{2+}-CaM simultaneously, as demonstrated by NMR spectroscopy. A 54-residue peptide that spans both the CaD1 and CaD2 sequences produced an NMR spectrum similar to that of the two shorter peptides. It has been suggested that

Fig. 5. CaM-binding domain sequences in some CaM target proteins. The numbers at the beginning and the end of each sequence correspond to the amino acid residue numbers in intact proteins. (A) The CaM-binding domains in skeletal muscle myosin light chain kinase (skMLCK) and smooth muscle myosin light chain kinase (smMLCK). Both domains bind to Ca^{2+}-CaM with two bulky hydrophobic residues separated by 12 residues anchoring the peptide to Ca^{2+}-CaM in an antiparallel orientation (Ikura et al. 1992; Meador et al. 1992). The conserved hydrophobic residues in these sequences are boxed. (B) The CaM-binding domain in CaM-dependent protein kinase II (CaMKII) and the first CaM-binding domain of caldesmon (CaD1). Both domains bind to Ca^{2+}-CaM with two bulky hydrophobic residues separated by eight residues anchoring the peptide to Ca^{2+}-CaM in an antiparallel orientation (Meador et al. 1993; Zhang and Vogel 1994*a*). The conserved hydrophobic residues in these sequences are boxed. (C) The amino acid sequence of melittin. The sequence of melittin is aligned from the C-terminal to the N-terminal, because melittin binds to Ca^{2+}-CaM in a parallel orientation, with the C-terminal region of melittin binding to the C-lobe of Ca^{2+}-CaM (Quiocho et al. 1997). (D) The two noncontiguous CaM-binding domains in phosphorylase kinase (Phkl3 and Phk5) and caldesmon (CaD1 and CaD2). Both proteins bind to Ca^{2+}-CaM with two noncontiguous parts simultaneously (Dasgupta et al. 1989; Trewhella et al. 1990; Huber et al. 1996; Zhou et al. 1997; Wang et al. 1997*b*). (E) The CaM-binding domains of neuromodulin and phophodiesterase 1A2 (PDE1A2). Both domains bind to CaM even in the absence of Ca^{2+} (Zhang et al. 1994*b*; Urbauer et al. 1995, 1996; T. Yuan, and H.J. Vogel, unpublished observations).

A:	skMLCK	^{342}K R R **W** K K N **F** I A **V** S A A N R **F** K K I S S S G A L^{367}
	smMLCK	^{796}A R R K **W** Q K T G H A **V** R A I G R **L** S S^{815}
B:	CaMKII	^{290}L K K F N A R R K **L** K G A **I** L T T M **L** A T R N F S^{314}
	CaD1	^{651}G V R N **I** K S M **W** E K G N **V** F S S^{667}
C:	Melittin	^{26}Q Q R K R K I W S I L A P L G T T L V K L V A G I G^{1}
D:	Phk13	^{302}G K F K V I C L T V L A S V R I Y Y Q Y R R V K P G^{327}
	Phk5	^{342}L R R L I D A Y A F R I Y G H W V K K G Q Q Q N R G^{367}
	CaD1	^{651}G V R N I K S M W E K G N V F S S^{667}
	CaD2	^{675}N K E T A G L K V G V S S R I N E W L T K T^{696}
E:	Neuromodulin	^{38}I Q A S F R G H I T R K K L^{51}
	PDE1A2	^{22}Q T E K M W Q R L K G I L R C L V K Q L^{41}

multiple contacts between Ca^{2+}-CaM and CaD are important for the formation of a productive interaction, and that Trp residues (Trp-659 and Trp-692) play a major role in this interaction (Huber et al. 1996; Graether et al. 1997; Wang et al. 1997*a*). Phosphorylase kinase and caldesmon are not the only two proteins that interact with Ca^{2+}-CaM through more than one CaM-binding domain. Other CaM-target proteins that have been reported to interact with Ca^{2+}-CaM at two distinct sites in their amino acid sequence are CaM-dependent cyclic nucleotide phosphodiesterase (PDElA1 and PDE1A2; Sonnenburg et al. 1995), the *N*-methyl-D-aspartate receptor (Ehlers et al. 1996), and the metabotropic glutamate receptor subtype 5 (Minakami et al. 1997), although it is not clear at present whether more than one CaM molecule is involved in the binding process. Hence, it seems that the two noncontiguous segments represent a separate class of CaM binding peptides. Determination of a high-resolution structure of such a complex will shed further light on its delicate interactions with Ca^{2+}-CaM.

CaM is a versatile regulatory protein because it not only can bind to target proteins in the presence of calcium but also can bind to some target proteins in the absence of calcium (Crivici and Ikura 1995; Vogel and Zhang 1995; Rhoads and Friedberg 1997). Neuromodulin is a neuronal protein that can interact with both membranes and (or) CaM. It binds to apo-CaM more tightly than to Ca^{2+}-CaM and it has been suggested to play a role in making CaM available in response to the increase in Ca^{2+} concentration near the membrane surface. It was demonstrated by TrNOE experiments that the neuromodulin peptide binds to apo-CaM with an α-helical structure (Zhang et al. 1994*b*). Subsequently, Urbauer et al. (1995) studied the interaction between apo-CaM and a Trp-substituted neuromodulin peptide (Phe-42-Trp) in detail by triple-resonance NMR spectroscopy. They found that binding of the peptide causes relatively small chemical shift changes in the spectra of apo-CaM with the larger chemical shift changes occurring in the C-lobe of apo-CaM. Structural analysis of apo-CaM has shown that the C-terminal domain of the protein adopts a semi-open conformation (Swindells and Ikura 1996). A V-shaped groove can be observed in this semi-open structure of apo-CaM (Kuboniwa et al. 1995; Zhang et al. 1995*a*). The crystal structure of the essential light chain (ELC) of scallop myosin has also shown a similar semi-open structure (Houdusse and Cohen 1995). In addition, the semi-open structure of ELC binds to the first six residues (IQxxxR) of the IQ motif of myosin heavy chain (Zhu and Ikebe 1994). Given the high structural similarity between apo-CaM and the ELC domain of myosin, it was proposed that apo-CaM might bind to the IQ motif of its targets primarily through the C-terminal domain of the protein (Swindells and Ikura 1996). More recently, Urbauer et al. (1996) used high-pressure NMR spectroscopy to separate the hydrophobic effects and ionic interactions between apo-CaM and neuromodulin peptide. This study indicated that the hydrophobic interaction dominates the peptide binding affinity, in agreement with what was observed in the structure of ELC of myosin (Houdusse and Cohen 1995). The authors proposed that the ionic interaction may determine the binding specificity of the initial encounter complex. A similar model has been suggested for the Ca^{2+}-CaM-smMLCK peptide complex on the basis of NMR hydrogen exchange experiments (Ehrhardt et al. 1995).

Two distinct directions remain for future structural studies of CaM-binding peptides. The first one is the determination

of the three-dimensional structures of novel binding motifs for CaM-peptide interactions, such as the structure of CaM in a complex with two noncontiguous peptides. Another direction is extending the structural studies to larger fragments of CaM's target proteins or even to intact target proteins. For example, a three-residue Glu-6-Glu-Gln-8 deletion in the N-terminal of CaM impairs its activation of either skMLCK or smMLCK (Persechini et al. 1996). This deletion mutation in CaM has little effect on the kinase binding affinity for CaM, and this is consistent with the notion that this region does not have extensive interaction with the skMLCK peptide or the smMLCK peptide in the NMR or X-ray structures (Ikura et al. 1992; Meador et al. 1992). The authors suggested that secondary interactions between Ca^{2+}-CaM and MLCK may be important to the activation of the kinase, but not to the binding of Ca^{2+}-CaM. In two recent studies, the CaM-binding domain sequences of neuronal nitric oxides synthase (nNOS) or endothelial nitric oxide synthase (eNOS) were exchanged with that of inducible nitric oxide synthase (iNOS) (Ruan et al. 1996; Venema et al. 1996). Both studies implied that regions other than the direct CaM-binding sequence (residues 503–532 in iNOS) are also important in conferring the Ca^{2+}-independent binding of iNOS to CaM. Edwards et al. (1998) reported on the activation of calcineurin and smMLCK by various CaM Met → Leu mutants. They concluded that the simple removal of an autoinhibitory domain from the active site of a target enzyme could not explain all of their data. They suggested that a conformational change in the phosphatase and kinase domains or additional interactions were involved in the activation process. Evidence for such a large-scale conformational change in MLCK upon binding CaM has recently been provided through small angle X-ray scattering studies of the complex (Krueger et al. 1997). Future determination of a high-resolution structure of an intact target protein complexed with CaM will greatly enhance our understanding of the function of the versatile protein CaM, as it activates target proteins.

Molecular basis of CaM's multitarget recognition

The most fascinating, yet perplexing, feature of CaM is that it can interact with more than 40 different targets, and that the CaM-binding domains, which are usually a continuous stretch of peptides with about 15–30 amino acid residues, do not show amino acid sequence homology. The only common feature of these peptide fragments is that they have a tendency to form basic, amphiphilic α-helices. Generally, in cases in which a regulatory protein or enzyme recognizes multiple targets or substrates, the targets or substrates share a high degree sequence homology. Examples of such interactions include SH3 domains recognizing polyproline sequence motif and proteases cleaving distinct specific amino acid sequences. Otherwise, protein–protein interactions are usually very specific, and generally one protein recognizes one target. It is interesting to note that the interaction between CaM and its targets is mediated exclusively via amino acid side-chain interactions. Most protein–protein contacts are a mixture of side chain – side chain, side chain – backbone, and backbone–backbone interactions, and hence the interaction mode seen in CaM is unique.

CaM contains an abnormally high amount of Met residues (9 Met out of a total of 148 amino acid residues), and 8 of the 9 Met residues are found in the hydrophobic surfaces of Ca^{2+}-CaM with 4 in each domain (Babu et al. 1988; Fig. 2). The four Met residues are located at the entrance of the hydrophobic pocket of each domain of Ca^{2+}-CaM. The base of the pocket is made up of aromatic (Phe) and branched aliphatic (Ire, Leu, and Val) amino acid residues and short Ala residues. In fact, the Met residues contribute to about 46% of the total exposed hydrophobic surface area of the protein (Babu et al. 1988). A surface rich in Met residues was proposed to allow CaM to bind many targets in a sequence-independent manner (Gellman 1991). The author pointed out that the existence of a sulfur atom in the unbranched side chain entails Met with unique flexibility and high polarizability. In addition, the high polarizability of the surfaces will not only allow the protein to interact with its target via London dispersion forces but also stabilizes the large solvent-exposed hydrophobic surfaces in the absence of targets. Indeed, in the refined crystal structure of Ca^{2+}-CaM, a number of ordered water molecules were observed in the C-terminal hydrophobic pocket of the protein. The N-terminal hydrophobic pocket was occupied by a highly polar ethanol molecule used in the protein crystallization (Chattopadhyaya et al. 1992). As expected, mutations of single or multiple flexible Met to rigid Leu residues do not drastically alter the structure of Ca^{2+}-CaM as these residues are all located on the surface of the protein (Zhang et al. 1994*c*; Edwards et al. 1998). Most single Met to Leu substitutions do not display altered target enzyme activation profiles. However, simultaneous mutation of all Met residues to Leu in the C-terminal domain created a mutant CaM with lower affinity for phosphodiesterase, MLCK, and calcineurin (Zhang et al. 1994*c*; Edwards et al. 1998). In addition, the replacement of Met residue with the less polarizable but isostructural unnatural amino acid norleucine leads to a mutant CaM with a reduced capacity to activate a number of enzymes such as MLCK and calcineurin (T. Yuan and H.J. Vogel, unpublished data). In a more recent study, the X-ray crystal structure of CaM complexed with a CaM-binding drug (W7) further demonstrated that the Met residues in the hydrophobic pocket of the protein can rearrange their side-chain conformation to accommodate targets with different sizes (Osawa et al. 1998). The properties of the Met side chains have been probed by ^{77}Se NMR of SeMet-CaM, and the outcome of these studies supports a role for the polarizability of S and Se (Zhang and Vogel 1994*b*).

NMR relaxation studies of the methyl groups have provided a picture of the dynamic properties of CaM in its various functional states. Deuterium (^{2}H) and Met methyl-^{13}C relaxation measurements have shown that the Met residues located at the entrances of the hydrophobic pockets are particularly flexible compared with other amino acid residues (Siivari et al. 1995; T. Hiraoki and H.J. Vogel, unpublished observations; R. Ishima, N. Farrow, L. Kay, M. Zhang, and M. Ikura, unpublished observations). In addition, the aliphatic and perhaps also the aromatic amino acids sitting at the bottom of the hydrophobic pockets are more rigid and

may serve as a scaffold of the binding pockets (R. Ishima, unpublished observations). The removal of Ca^{2+} leads to an overall increase in the rigidity of the Met as well as other methyl-containing amino acid side chains, as most of these amino acids become buried in the apo-form of the protein. In the complex of CaM with the skMLCK peptide, about 80% of the methyl groups in the hydrophobic pockets of CaM make direct contacts with the peptide, and some of these experience a reduced motion in the complex. The use of Met-rich hydrophobic surface area to recognize multiple targets in a sequence-independent manner has also been observed in other biological systems such as the 54-kDa subunit of the signal recognition particle and signal peptides (Bernstein et al. 1989), and such interactions may be a general theme in protein–protein interactions.

In addition to the two Met-rich hydrophobic surfaces, the flexible central linker region in Ca^{2+}-CaM also plays a very important role in CaM's functional versatility. This linker region is sufficiently flexible to allow Ca^{2+}-CaM to bind to basic, amphiphilic α-helices with two bulky hydrophobic residues separated by either 8 or 12 residues (Ikura et al. 1992; Meador et al. 1992, 1993; Zhang and Vogel 1994*a*). Moreover, the central linker region of Ca^{2+}-CaM can be extended to let Ca^{2+}-CaM interact with two noncontiguous sequences in phosphorylase kinase (Trewhella et al. 1990). The central linker region in Ca^{2+}-CaM is capable of adjusting the relative orientation of the N- and C-lobe of Ca^{2+}-CaM to provide maximal contact with the Met-rich hydrophobic surfaces for target protein binding. Tabernero et al. (1997) recently reported an X-ray structure of a Ca^{2+}-CaM mutant in which both Thr-79 and Asp-80 in the central linker region are deleted. This mutant protein changes the relative orientation of the N- and C-lobe in Ca^{2+}-CaM, making the C-lobe rotate 220° relative to the N-lobe. Consequently, the two hydrophobic patches are closer to each other and less accessible to target protein binding (Tabernero et al. 1997). The activation of phosphodiesterase by this mutant was severely impaired (VanBerkum et al. 1990). A recently published X-ray structure of four Ca^{2+}-bound troponin C also supports the above notion (Houdusse et al. 1997). Troponin C is also a Met-rich Ca^{2+}-regulatory protein, yet it only binds to one component (troponin I) in vivo. Houdusse et al. (1997) found that the flexibility of the central linker region in $Ca^{2+}{}_4$-troponin C is different from the one in Ca^{2+}-CaM. When the central linker sequence ^{85}EDAKGK^{90} in skeletal muscle troponin C was replaced by the CaM-sequence DTD, this troponin C mutant was able to fully activate phosphodiesterase in vitro (Gulati et al. 1993). Thus, in addition to the Met-rich regions, the unique properties of CaM's central linker region are critical for CaM's versatility.

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