Peptide Binding by a Fragment of Calmodulin Composed of EF-Hands 2 and 3†

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ABSTRACT: Calmodulin (CaM) is composed of two EF-hand domains tethered by a flexible linker. Upon Ca\(^{2+}\)-binding, a fragment of CaM encompassing EF-hands 2 and 3 (CaM2/3; residues 46–113) folds into a structure remarkably similar to the N- and C-domains of CaM. In this study, we demonstrate that Ca\(^{2+}\)-ligated CaM2/3 can also bind to a peptide representing the CaM-recognition sequence of skeletal muscle myosin light chain kinase (M13) with an equimolar stoichiometry and a dissociation constant of 0.40 ± 0.05 μM. On the basis of an analytical ultracentrifugation measurement, the resulting complex exists as an equilibrium mixture of 2:2 heterotetrameric and 1:1 heterodimeric species. Chemical shift perturbation mapping indicates that, similar to CaM, the peptide associates with a hydrophobic groove crossing both EF-hands in CaM2/3. However, upon binding the M13 peptide, many residues in CaM2/3 yielded two equal intensity NMR signals with the same 15N relaxation properties. Thus, the 2:2 CaM2/3-M13 tetramer, which predominates under the conditions used for these studies, is asymmetric with each component adopting spectroscopically distinguishable conformations within the complex. CaM2/3 also weakly stimulates the phosphatase activity of calcineurin and inhibits stimulation by native CaM. These studies highlight the remarkable plasticity of EF-hand association and expand the diverse repertoire of mechanisms possible for CaM-target protein interactions.

Calmodulin (CaM\(^{1}\)) is a highly conserved, ubiquitous EF-hand protein involved in the regulation of more than 100 target proteins in response to Ca\(^{2+}\) signals (1, 2). Rather than a cell having many proteins, each with a Ca\(^{2+}\)-sensing domain, CaM has evolved as a common mediator that can detect a Ca\(^{2+}\) stimulus and respond by activating a diverse set of target proteins. Furthermore, the interaction between CaM and these targets is generally strong, with dissociation constants on the order of 0.01–100 nM (3). In this respect, CaM is exceptional in the biological world because of its high affinity, yet broad specificity, for target recognition (1).

The basic principles underlying Ca\(^{2+}\) binding to CaM and its subsequent recognition of CaM-target proteins have been well established (4–6). CaM contains four Ca\(^{2+}\)-binding helix-loop-helix EF-hand motifs, with EF-1 and -2 associating to form its N-domain, and EF-3 and -4 forming its C-domain. The two domains are tethered through a flexible intervening linker sequence. Upon Ca\(^{2+}\) binding, the helices within each EF-hand shift from roughly antiparallel to a more perpendicular orientation, thereby exposing a hydrophobic pocket in each domain of CaM to which specific hydrophobic anchor residues in the recognition sequence of a CaM-target can bind (6). The flexibility of the CaM linker region allows the N- and C-domains to bind different hydrophobic residues within a variety of recognition sequences, thereby contributing to its high affinity of association with a diverse set of target proteins (1). These recognition sequences are often part of an auto-inhibitory domain or a pseudo-substrate motif. Thus, the activation of a downstream biological response typically results from the relief of target auto-inhibition upon CaM binding. Although target recognition generally involves both EF-hand domains of CaM, previous studies have shown that its N- and C-domain tryptic fragments of CaM can also bind to target sequences, acting as agonists or antagonists of specific CaM-stimulated enzymes (7–10).

Recently, we characterized a fragment of CaM composed of EF-2 and -3 (residues 46–113 (CaM2/3)) by NMR and CD spectroscopy (11). Upon the sequential binding of 2 equivalents of Ca\(^{2+}\) (K\(_{d1}\) = 30 ± 5 μM to EF-3 and a K\(_{d2}\) >
1 mM to EF-2), this peptide folds into a globular, monomeric structure that is strikingly similar to both the N- and C-domains of Ca\textsuperscript{2+}-ligated CaM. Importantly, this folding involves the non-native pairing of EF-2 and -3, with the linker sequence of CaM now serving as a flexible loop joining these two Ca\textsuperscript{2+}-binding motifs. Consistent with its structural similarity to the domains of Ca\textsuperscript{2+}-ligated CaM, CaM2/3 possesses a solvent exposed hydrophobic groove. This suggested that CaM2/3 might also bind to and activate CaM-targets.

In this study, we demonstrate that CaM2/3 can bind to a peptide (M13) representing the CaM recognition sequence of skeletal myosin light chain kinase (skMLCK). The NMR resonances from the mainchain H, 13 C, and 15 N nuclei in M13-bound CaM2/3 were partially assigned using NMR spectroscopy. Although insufficient for a complete structural analysis, these assignments confirmed that the peptide binds to the hydrophobic groove of CaM2/3. However, many amides in CaM2/3 yielded double 1 H-15 N HSQC resonances of equal intensity upon the formation of the CaM2/3-M13 complex, indicating the presence of two conformations in slow exchange on the chemical shift time scale. Analytical ultracentrifugation (AUC) experiments demonstrate that this complex exists in an equilibrium between 2:2 and 1:1 complexes with a dissociation constant of 130 ± 30 μM. Therefore, at the concentrations under which the NMR experiments were performed (between 0.1 and 0.5 mM), CaM2/3-M13 exists primarily as a 2:2 heterotetramer. Furthermore, on the basis of the equal intensity double resonances, this 2:2 CaM2/3-M13 complex must be asymmetrical.

In addition to binding the M13 peptide, CaM2/3 can also interact with calcineurin, as evidenced by both its ability to weakly stimulate the phosphatase activity of this enzyme and to inhibit simulation of calcineruin by native CaM. These studies exemplify the remarkable plasticity of EF-hand sequences to associate into EF-hand domains and mediate Ca\textsuperscript{2+}-dependent recognition of target proteins in biological signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Synthesis of the M13 Peptide.** The 26 residue M13 peptide, KRRWKKNFIAVSAANRFKKISSGAGL, corresponding to the CaM recognition sequence of skMLCK, was synthesized via solid-phase peptide synthesis on an Applied Biosystems 433A peptide synthesizer (12, 13), cleaved (14), and purified by RP-HPLC according to previously described protocols (15). The identity of the M13 peptide was confirmed by electrospray LC-MS (observed, 2963.3 Da; predicted, 2963.5 Da) and quantitated by peptide hydrolysis followed by amino acid analysis as described above. Secondary structure predictions were obtained from 13 C\textsuperscript{a} versus 13 C\textsuperscript{b} chemical shift differences relative to random coil values (secondary chemical shifts), calculated for each residue i in M13-bound CaM2/3 as Δδ(i) = (δ\textsuperscript{13}C\textsuperscript{a}(i) − δ\textsuperscript{13}C\textsuperscript{b}(i)) − (δ\textsuperscript{13}C\textsuperscript{a}(coil) − δ\textsuperscript{13}C\textsuperscript{b}(coil)) and weighted with nearest-neighbor values, Δδ(i) + 2Δδ(i+1) + Δδ(i−1))/4, using the program CSI (23–25). The M13 binding site was identified from differences in the combined 1 H,15 N/13 C chemical shifts between corresponding amide, aromatic, or alanine methyl groups in CaM2/3 and the CaM2/3-M13 complex, calculated as Δδ = [(Δδ\textsuperscript{1}H)\textsuperscript{2} + (Δδ\textsuperscript{1}H/δ\textsuperscript{15}N/δ\textsuperscript{13}C)]\textsuperscript{1/2}, where I is either 13 C or 15 N, and γ is the gyromagnetic ratio for the appropriate nuclei. These ratios are 26.75, -2.71, and 6.73 x 10\textsuperscript{7} rad T\textsuperscript{-1} s\textsuperscript{-1} for 1 H, 15 N, and 13 C, respectively.

**NMR-Monitored Titration of CaM2/3 with M13.** The binding of unlabeled M13 peptide to 15 N-CaM2/3 was monitored with sensitivity enhanced gradient 15 N-HSQC spectra run at 25 °C on a Varian Inova 600 MHz spectrometer. A total volume of 85 μL of 1.8 mM M13 peptide in NMR buffer was added in 5 or 10 μL aliquots to a sample of 15 N-CaM2/3, initially at 0.18 mM in 0.48 μL of the same buffer. At the end of the titration, there was a 1:8:1 molar ratio of M13:15 N-CaM2/3. The data were analyzed by fitting 15 N-CaM2/3 amide peak intensity, after correction for dilution, versus the concentration of added M13, followed by 11/2 inspection of target proteins in biological signaling pathways.
by fitting to a single site binding model using the program CalLigator (26).

**Backbone 15N Relaxation of the CaM2/3-M13 Complex.** Backbone amide 15N relaxation parameters were acquired for 0.5 mM M13-bound 13C/15N-CaM2/3 at 25 °C using a Varian Unity 500 MHz NMR spectrometer (27, 28). Data points for the T2 (10 to 1053.5 ms) and T1 (16.7 to 166.0 ms) experiments were collected in random order. Steady-state heteronuclear 1H(15N)NOE spectra were acquired with and without 2 s of 1H saturation and a total recycle delay of 5 s. All data were processed with NMRPipe (21) and T1 and T2 lifetimes obtained by fitting to a single-exponential decay using Sparky (22). Errors for heteronuclear 1H(15N)NOE ratios were estimated according to previously described methods (28). The effective correlation time for isotropic rotational diffusion (τc) of the CaM2/3-M13 complex and the model-free order parameters (S2) for the backbone amides of bound CaM2/3 were calculated with Tensor 2.0 (29). Residues for which the 1H(15N)NOE ratios were <0.6 and for which the values of ([(R2) - R0]/(R2)) - (R0 - R1)/(R0)] > 1.5 times the standard deviation of this difference were excluded from the τc calculations because of the possibility of fast internal motions or chemical exchange broadening (30).

**Analytical Ultracentrifugation.** Sedimentation velocity experiments were conducted at 20 °C with a Beckman Optima XL-I analytical ultracentrifuge equipped with both absorbance and interference optics. Standard aluminum Optima XL-I analytical ultracentrifuge equipped with both points for the Peptide Binding by Calmodulin EF-Hands 2 and 3 Biochemistry, Vol. 46, No. 29, 2007

isotropic rotational diffusion (τc) of the solution of the Lamm equation, calculated with SEDFIT (34), assuming the regularization parameter p to be 0.95. Sedimentation coefficient increments of 200 were used in the appropriate range for each sample.

**Calcineurin Stimulation Assay.** The stimulation of calcineurin (protein phosphatase-2B or PP-2B) by both CaM and CaM2/3 was assessed in 96 well plate format by measuring phosphorylase activity against p-nitrophenol phosphate (pNPP). The reaction buffer was composed of 50 mM Tris at pH 7.0, 1 mM CaCl2, 1 mM NiCl2, and 0.125 mg/mL BSA. Samples with calcineurin alone as well as calcineurin with CaM2/3 or CaM were preincubated at 37 °C for 30 min. The reaction was initiated by the addition of pNPP, in reaction buffer, to a final concentration 0.9 mg/mL. The appearance of nitrophenol was quantitated with a Labsystems Multiskan Ascent plate reader at 405 nm (35, 36). Initial rates were measured from reactions with 50 nM (0.70–0.74 μg) calcineurin, and CaM or CaM2/3 at concentrations of 0, 5, 10, 25, 50, 100, and 300 nM per reaction. A competition assay was also performed with 0, 10, 50, and 300 nM CaM samples containing 0, 10, 50, and 100 nM CaM2/3.

**RESULTS**

**Binding of M13 to CaM2/3 Measured by NMR-Monitored Titrations.** The interaction between Ca2+-saturated 15N-CaM2/3 and M13 was investigated by 15N-HSQC spectroscopy. The chemical shifts and relaxation properties of an amide are highly sensitive to even subtle conformational changes such as those occurring at interaction surfaces. Thus, shift perturbations in NMR-monitored titrations can be used to qualitatively identify binding interfaces and to quantitatively measure binding affinities. Upon the incremental addition of M13, a progressive disappearance of the amide 1H,15N signals from unbound CaM2/3 was observed, along with concomitant appearance of a new set of signals (Figure 1). These signals, which arise from the CaM2/3-M13 complex, indicate that binding occurs in the slow exchange limit on the chemical shift time scale (i.e., the rate of exchange between free and bound states is less than the frequency differences for corresponding amides in each state).

A striking feature of the 15N-HSQC spectrum of M13-saturated CaM2/3 is the appearance of more amide signals than expected and observed with free CaM2/3. Using conventional 1H-13C-15N correlation experiments recorded on an equimolar complex of 15N/13C-CaM2/3 with unlabeled M13, resonances from the main chain nuclei of the labeled protein were assigned. As summarized in Figure 1, 33 out of 63 non-proline residues in M13-bound CaM2/3 yielded two resolved amide 1H-15N peaks of equal intensity, arbitrarily denoted as ‘a’ and ‘b’. Similarly, additional peaks were observed in the 13C-HSQC spectrum of the labeled protein. Two resolved indole 1H signals of equal intensity from the sole tryptophan in M13 were also present in the 1H NMR spectrum of the CaM2/3-M13 complex (not shown). Thus, CaM2/3-M13 must adopt at least two distinguishable conformations of very similar or identical populations. Unfortunately, because of spectral degeneracy, we were unable to specifically determine which of the ‘a’ and ‘b’ signals for a given amide corresponds to which conformation of the bound CaM2/3. That is, many residues with two amide signals still had similar 13C and 15N shifts at the recorded spectral resolution, reflecting the generally lower sensitivity of alphatic resonances versus amide resonances to conformational perturbations. Also, because of spectral complexity, we were unable to confidently assign the resonances from the unlabeled M13 peptide (except for its indole 1H(1H)) using 15N/13C-filtered 1H-1H TOCSY and NOESY experiments in which signals from 15N/13C-CaM2/3 were suppressed. Finally, we did not detect the transfer of magnetization between the ‘a’ and ‘b’ peaks of any given amide in 1H-15N-1H NOESY-HSQC and HSQC-monitored N2 exchange experiments (not shown) (37). Thus, any exchange between the
bound states of the CaM2/3-M13 complexes, either intramolecularly or via dissociation to free M13 and CaM2/3, occurs on a time scale slower than their $T_1$ values (i.e., seconds).

A quantitative analysis of the titration of CaM2/3 with the M13 peptide is presented in Figure 2. On the basis of a plot of the average normalized intensities of the amide $^{1}H$-$^{15}N$ peaks from residues 48–55, 65–92, and 102–113 within the helical and linker regions of bound CaM2/3, saturation occurs at an equimolar CaM2/3/M13 ratio (Figure 2A). However, as demonstrated below, this corresponds to a 2:2 heterotetrameric complex. Fitting of these titration data to a model describing the equilibrium CaM2/3 + M13 ⇌ CaM2/3-M13 yielded an apparent $K_d$ of 0.40 (±0.05) μM.

Note that this value, which is consistent with relatively high affinity association in the slow exchange regime, reflects the formation of all bound species and the assumption of equivalent binding sites in the 2:2 complex. Figure 2B also plots the absolute intensities from three residues showing double resonances F65 (F65a and b), I100 (I100a and b), and L105 (L105a and b) versus the concentration ratio of added M13 to $^{15}$N-CaM2/3. Over the entire titration experiment, the observed double peaks of these, and all other amides (not shown), are of nearly equal intensity. Hence, the two NMR-distinguishable conformations of the CaM2/3-M13 complex are isoenergetic, forming with the same binding affinity.

Analytical Ultracentrifugation of the CaM2/3-M13 Complex. Analytical ultracentrifugation (AUC) was used to further investigate the stoichiometry of the CaM2/3-M13 complex. Sedimentation velocity and equilibrium data were obtained for CaM2/3, M13, and CaM2/3-M13 in the presence of excess Ca$^{2+}$ at 20 °C. As summarized in Figure 3, the M13 peptide (75 μM) sedimented as an essentially monodisperse species with a sedimentation coefficient of 0.48 S. Consistent with this value, a global fit of the sedimentation equilibrium experiments, performed at three different concentrations, confirmed that the M13 peptide is monomeric under these experimental conditions (apparent MW = 2810 Da; theoretical = 2963 Da). In contrast, fitting of the sedimentation velocity data measured for CaM2/3 (75 μM) revealed a major 1.3 S species, as well as a minor ~2.4 S species (6%) attributable to monomeric and dimeric forms of the protein, respectively. Sedimentation equilibrium analysis confirmed that CaM2/3 has a weak tendency to dimerize ($K_d$ ~ 4 mM). Therefore, at submillimolar concentrations, CaM2/3 is predominantly monomeric, as demonstrated previously through NMR structural and dynamic studies (11).

Sedimentation velocity data were also recorded for the complex of CaM2/3 and M13 (each at 38, 75, or 150 μM). Given the apparent $K_d$ for complex formation of 0.40 μM, >90% of CaM2/3 and M13 should be in the bound form under the conditions of these experiments. For each sample, the fit c(s) distribution revealed a major species with a sedimentation coefficient of 2.1 S (Figure 3). This sedimentation coefficient is larger than that expected for a 1:1 CaM2/3-M13 complex, but smaller than that for a 2:2 heterotetramer.
Specifically, a sphere with the same MW as that of the 1:1 complex would have an $S_{\text{max}}$ of 1.6 S, whereas that corresponding to a 2:2 complex would have an $S_{\text{max}}$ of 2.3 S (assuming $f/f_0 = 1.2$ due to hydration; asymmetry would reduce these values). Therefore, the CaM2/3-M13 complex exists as an equilibrium between 2:2 and 1:1 species. Alternative species such as a 2:1 CaM2/3-M13 complex are excluded on the basis of the equimolar equivalence point found in the NMR-monitored titrations (Figure 2A).

Following upon this result, equilibrium sedimentation measurements were carried out at three rotor speeds and with CaM2/3 and M13 at 38, 75, or 150 $\mu$M each. As summarized in Table 1, the apparent weight-averaged molecular weight of the sample increased with increasing concentrations or reduced rotor speeds. The lowest apparent molecular weight (13.7 kDa) measured is closer to, but still larger than, the expected molecular weight of a 1:1 CaM2/3-M13 complex (10.6 kDa), and the highest (20.1 kDa) is close to that expected for a 2:2 complex (21.3 kDa). Furthermore, the variation of apparent molecular weight with sample concentration reflects the reversibility of the association. This behavior suggests that the higher apparent molecular weight species is due to the self-association of a 1:1 CaM2/3-M13 complex to a 2:2 CaM2/3-M13 complex, rather than the formation of nonspecific aggregates. Moreover, assuming that the CaM2/3 and M13 are fully bound to one another, we were able to globally fit all nine data sets, with SEDPHAT (33), for an equilibrium of the form $(\text{CaM2/3-M13})_2 \rightleftharpoons 2(\text{CaM2/3-M13})$ to obtain an apparent dissociation constant of 130 $\pm$ 30 $\mu$M.

#### Table 1: Equilibrium Sedimentation of the CaM2/3-M13 Complex

<table>
<thead>
<tr>
<th>conc ($\mu$M)</th>
<th>apparent molecular weight (kDa) for a given rotor speed (rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaM2/3, M13</td>
<td>20,000, 30,000, 50,000</td>
</tr>
<tr>
<td>150, 150</td>
<td>20.1, 18.0, 14.5</td>
</tr>
<tr>
<td>75, 75</td>
<td>17.9, 17.7, 14.5</td>
</tr>
<tr>
<td>38, 38</td>
<td>16.7, 16.7, 13.7</td>
</tr>
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</table>

*Total concentration of each species. With $K_d = 0.40 \mu$M, >90% of CaM2/3 and M13 should exist in equimolar complexes under these conditions.*

Concentration-Dependent NMR Spectra of the CaM2/3-M13 Complex. On the basis of the AUC results, the CaM2/3-M13 complex exists predominantly as a 2:2 heterotetramer under the conditions used for the NMR spectral assignments. The observed sets of two equal intensity signals for many residues can thus be explained if the complex is internally asymmetric, with both CaM2/3 and M13 adopting two spectroscopically distinct conformations. To further investigate the stoichiometry of the CaM2/3-M13 complex, we recorded NMR spectra as a function of sample concentration. Between 500 and 100 $\mu$M, the $^1$H-$^15$N-HSQC spectra of $^15$N-labeled CaM2/3 in the presence of equimolar M13 remained
invariant, without changes in the chemical shifts or relative peak intensities of any amides. However, upon dilution to 25 μM, a new set of dispersed $^1$HN,$^{15}$N signals, distinct from those of the tetramer or free CaM2/3, appeared (not shown). Because of the low sample concentration, the new peaks were not assigned. However, for amides with the most dispersed resonances, only single new peaks were observed. Thus, we attribute these signals to a 1:1 CaM2/3-M13 complex. The lack of these signals in samples more concentrated than 100 μM suggests that the actual tetramer–dimer dissociation constant is lower than the fit value of 130 ± 30 μM (i.e., 500 μM total complex would partition as ~70% tetramer and ~30% dimer with this $K_d$). This discrepancy can be attributed to difficulties in fitting equilibrium ultracentrifugation data to the mixed species present (i.e., free CaM2/3 monomers or possibly dimers and M13 as well as the CaM2/3-M13 complexes).

**Secondary Structure of the CaM2/3-M13 Complex.** Because of the lack of assignments for the bound M13 peptide and the inability to assign the ‘a’ and ‘b’ signals from CaM2/3 to specific conformations, we could not determine the tertiary structure of the CaM2/3-M13 complex. However, the secondary structure of M13 bound CaM2/3 can be accurately predicted using chemical shift information. For example, $^{13}$C$^\alpha$ chemical shifts move downfield/upfield for residues in $\alpha$-helices/β-strands relative to a random coil state, whereas the reverse holds for $^{13}$C$^\beta$ chemical shifts (38). On the basis of observed versus reference $^{13}$C$^\alpha$,$^{13}$C$^\beta$ chemical shift differences, the two spectroscopically distinguishable forms of the CaM2/3-M13 complex have very similar secondary structures to one another and, with the exception of the linker region, to that of unbound CaM2/3 (Figure 4) (11). In particular, four common $\alpha$-helical regions and two β-strands could be identified in all forms of CaM2/3. This conclusion is also supported by the partial analyses of a $^1$H-$^1$N-$^1$H NOESY-HSQC spectrum, in which diagnostic NOE interactions were detected between I63 $^1$HN and I100 $^1$HN in free CaM2/3 and in both forms (I63a/b, and I100a/b) of the CaM2/3-M13 complex (data not shown). Together these data confirm that the antiparallel β-sheet between EF-2 and -3 in CaM2/3 is also retained in the M13 complex.

In addition to the invariant $\alpha$-helices and β-sheet, residues 75–79 in M13-bound CaM2/3 show $^{13}$C$^\alpha$,$^{13}$C$^\beta$ chemical shift differences indicative of a new, short $\alpha$-helical segment. In free CaM2/3, these residues, which correspond to the interdomain linker in native CaM (22)ARKMKD(TD38) (39), form a flexible loop bridging EF-2 and -3 (11). Thus, M13 binding is accompanied by a conformational change in CaM2/3 that includes induction of a helix in the linker sequence.

**CaM2/3 Chemical Shift Perturbations due to M13 Binding.** The differences in chemical shifts between corresponding nuclei in free and M13-bound CaM2/3 ($\Delta\delta$) provide a qualitative identification of the residues undergoing conformational changes upon complex formation. As shown in Figure 1 and quantitated in Figure 5A, most amides in CaM2/3 exhibited detectable shift perturbations upon complex formation, with an average combined $^1$HN and $^{15}$N $\Delta\delta$ of 0.27 ± 0.23 ppm. This is suggestive of global tertiary conformational changes. However, the largest amide shift perturbations occur for residues R74, K75, M76, and D78 within the linker sequence and M71, M72, E82, E83, F92, R106, M109, T110, and N111 in helices D, E, and F. The pronounced amide shift changes for the linker residues 74–78 are consistent with the formation of a helical segment in this region of CaM2/3, as noted above. Spectral differences between CaM2/3 and the CaM2/3-M13 complex were also calculated for the $^1$H and $^{13}$C nuclei in aromatic residues as well as the alanine methyls. An examination of the aromatic $^1$C$^\beta$-HSQC spectrum of the CaM2/3-M13 complex showed that the $^1$H$^\beta$ and $^{13}$C$^\beta$ chemical shifts of F65, Y99, and H107 are very similar to those of the unbound protein, whereas F68, F89, and F92 were significantly perturbed (i.e., $\Delta\delta$ > 0.3 ppm) (Figure 5B). Adjacent to these latter aromatics, A73 and A88 also exhibited large $^1$H$^\beta$ and $^{13}$C$^\beta$ chemical shift changes (i.e., $\Delta\delta$ > 0.20 ppm) upon M13 binding (Figure 5C).

The chemical shift perturbations accompanying the formation of the CaM2/3-M13 complex are mapped onto the structure of the unbound protein in Figure 6A and B. These changes could be due either to direct interactions with M13 or a second CaM2/3 within the 2:2 complex or to indirect structural perturbations. Without assignments of the putative 1:1 complex under dilute conditions, the former possibilities cannot be distinguished. With this caveat in mind, it is striking that residues showing the largest amide, aromatic, or alanine methyl chemical shift changes clearly map to a
hydrophobic groove that crosses EF-2 and -3. Analogous to the N- and C-domains of CaM (6), this strongly suggests that the M13 peptide binds within this groove via hydrophobic interactions, augmented by additional contacts with flanking polar and charged groups. The chemical shift differences between the 'a' and 'b' double peaks in the CaM2/3-M13 complex are plotted similarly onto the surface of unbound CaM2/3 in Figure 6.

CaM2/3-M13 Complex Backbone Dynamics from Amide 15 N Relaxation. The global and local backbone dynamic properties of the CaM2/3-M13 complex were investigated

by 15N T1, T2, and heteronuclear 1H(15N)NOE relaxation measurements (Figure 7). Consistent with its larger size due to the bound peptide, the CaM2/3-M13 complex exhibits longer average T1 (0.53 ± 0.07 s) and shorter average T2
(0.085 ± 0.02 s) values than unbound CaM2/3 (average $T_1 = 0.41 ± 0.07$ s and $T_2 = 0.13 ± 0.03$ s (II)). Excluding residues with $T_1/T_2$ or heteronuclear $^1$H-$^{15}$N NOE ratios indicative of conformational exchange or a high degree of internal mobility, analysis of relaxation data yielded an effective correlation time ($\tau_c$) of 7.7 ± 0.1 ns for the global isotropic tumbling of the CaM2/3-M13 complex. This is longer than that measured for CaM2/3 alone (5.1 ± 0.2 ns (II)) yet somewhat shorter than that of 9.9 ns reported for a CaM-smMLCK complex at 28 °C (a complex similar in size to the proposed 2:2 CaM2/3-M13 heterotetramer) (40). While recognizing that both shape and size contribute to rotational diffusion, the $^{15}$N relaxation data may be more consistent with a 1:1 CaM2/3-M13 complex. However, for amides with two sets of $^1$H-$^{15}$N signals in the CaM2/3-M13 complex, the ‘a’ and ‘b’ peaks exhibited very similar $T_1$ and $T_2$ lifetimes. Therefore, it is unlikely that one set of signals corresponds to a 1:1 complex and the other to a 2:2 complex in slow exchange on the chemical shift time scale, as the higher molecular weight of the latter would systematically yield increased $T_1$ and decreased $T_2$ values for one set relative to the other. Rather, the ‘a’ and ‘b’ peaks must arise from similar sized and equally populated species, which is most consistent with CaM2/3-M13 existing predominantly as an asymmetric 2:2 complex under the conditions used for relaxation measurements.

The internal dynamic properties of the backbone of CaM2/3 bound to M13 were described using the isotropic Lipari–Szabo model-free formalism in terms of a generalized order parameter $S^2$ that decreases from 1 to 0 with increased mobility of the amide $^1$H-$^{15}$N (Figure 7D). The CaM2/3-M13 complex exhibits relatively uniform $S^2$ values of 0.90 ± 0.07, consistent with a well-folded structure. Slightly reduced $S^2$ values are noted, for example, with residues in the loop regions between helices C/D and E/F of the EF-2 and -3 motifs, respectively. More interestingly, the changes in the order parameters ($\Delta S^2 = S^2_{CaM2/3-M13} - S^2_{CaM2/3}$) of CaM2/3 due to M13 binding are presented in Figure 7F. Most notably, the $S^2$ values for residues near the C-terminus of the protein, within helices C, E, and F, and within the linker are higher in the complex than in the free protein, indicative of dampened backbone mobility. In the case of the linker, this is consistent with the $^{13}$C$\alpha$-$^{13}$C$\beta$ secondary chemical shift analyses showing the formation of a helix upon binding (Figure 4). In contrast, the amide order parameters for segments within both EF-hands of CaM2/3 decreased upon complex formation. At face value, this suggests an increase in backbone mobility for some regions of CaM2/3. Alter-
the result of conformational exchange broadening (\( \tau_1 \) not unbound, state (\(-\)). This relaxation behavior is usually the result of conformational exchange broadening (\( R_{ex} \)) due to intermediate time scale (\( ms - \mu s \)) motions (Figure 7E). Several of these residues are perturbed upon the binding of CaM2/3 to M13, and thus, their amide relaxation may report mobility of CaM2/3 and/or the M13 peptide at this interfacial region.

**Calcineurin Activity Assay.** The M13 binding activity displayed by CaM2/3 suggests that it may also retain some of the function of native CaM such as the stimulation of calcineurin phosphatase activity. The results of a phosphatase assay (36) confirm that CaM2/3 produces a small, but significant, increase in the activity of calcineurin (Figure 8A).

Furthermore, CaM2/3 also inhibited the stimulation of calcineurin by native CaM. This is evidenced by a CaM2/3 concentration-dependent reduction in the apparent maximal rate of calcineurin phosphatase activity stimulated by CaM (Figure 8B).

**DISCUSSION**

To investigate the structural and functional diversity possible for EF-hand proteins, we characterized a fragment of CaM composed of EF-2 and -3 and the intervening linker sequence. Although Ca\(^{2+}\)-free CaM2/3 is predominantly unfolded, upon binding two equivalents of Ca\(^{2+}\), the protein adopts a structure that is strikingly similar to that of either the N- or C-terminal domain of Ca\(^{2+}\)-ligated CaM (II). In particular, CaM2/3 has a hydrophobic groove resembling those that mediate the binding of CaM-target sequences to both domains of Ca\(^{2+}\)-ligated CaM. Therefore, we hypothesized that the non-natively paired EF-hands of CaM2/3 could also bind such targets. Using NMR spectroscopy, analytical ultracentrifugation, and enzymatic assays, we have confirmed this hypothesis for the M13 peptide and calcineurin.

**CaM2/3-M13 Forms an Asymmetric Heterotetramer.** \( ^{15}\)N-HSQC-monitored titrations revealed that \( ^{15}\)N-CaM2/3 binds the M13 peptide with high overall affinity (\( K_d = 0.40 \pm 0.05 \mu M \)). This dissociation constant is comparable to those of 0.3 and 3 \( \mu M \) reported for the binding of the tryptic N- and C-domain fragments of CaM to skMLCK, respectively (41). However, the observed equimolar stoichiometry excludes the possibility that two CaM2/3 molecules associate with one M13, forming an asymmetric 2:1 CaM2/3-M13 complex and thereby recapitulating the roles of the N- and C-domains of CaM. Furthermore, in contrast to CaM, which yields a single M13 complex (6), two sets of equally intense amide \( ^1H \)-\( ^{15}\)N and aliphatic/aromatic \( ^1H\)\(^{13}\)C signals were observed for many residues in the CaM2/3-M13 complex. The sole tryptophan from M13 also yielded two indole signals, which provides additional evidence against a 2:1 species. Although this spectral doubling could arise from CaM2/3 and M13 forming conformationally distinct 1:1 heterodimers, such a situation seems unlikely because the two species would have to be isoenergetic in order to be present at equal populations.

Complementing these spectroscopic results, AUC data revealed that the CaM2/3-M13 complex exists as an equilibrium between a 1:1 heterodimer and an 2:2 heterotetramer with an apparent dissociation constant of 130 ± 30 \( \mu M \). Thus, under the conditions used for most of the NMR experiments, the heterotetramer predominates, and only upon dilution of the complex to 25 \( \mu M \) were a new set of dispersed NMR signals attributable to the heterodimer observed. Although the global isotropic tumbling time (\( r_t \)) of CaM2/3-M13 at 0.5 \( mM \) is somewhat shorter than that expected for a 21.3 kDa species (42), the fact that the ‘a’ and ‘b’ signals for each residue exhibited very similar \( ^{15}\)N relaxation properties argues strongly that both arise from similar sized species (i.e., not a mixture of 1:1 and 2:2 oligomers).

Combining the results from the NMR and AUC measurements, we conclude that the CaM2/3-M13 complex adopts two spectroscopically distinct conformations as part of an
internally asymmetric 2:2 heterotetramer. Exchange between these equally populated conformations, and with free CaM/M-
3, occurs on a time scale longer than seconds, as shown by the distinct chemical shifts of all three species during a
titration experiment. Unfortunately, because of spectral overlap and the lack of assignments for the putative het-
erodimer, we were unable to determine the three-dimensional structure(s) of the CaM/M-3/M13 complex(es) by standard
NMR methods or to identify the CaM/M-3–CaM/M-3 interface within the 2:2 species. Thus, the exact nature of the
conformational differences leading to the asymmetry of the CaM/M-3/M13 complex remains to be established.

M13 Binds to a Hydrophobic Groove in CaM/M-3. Chemi-

cal shift perturbation mapping demonstrates that the M13 peptide binds to the hydrophobic groove identified in the
NMR-derived structure ensemble of CaM/M-3 (Figure 6). In particular, the greatest amide, aromatic, and alanine methyl
chemical shift changes occur for residues in the linker region and in helices D, E, and F. These residues include F68, M71,
M72, A73, M76, A88, F89, F92, and M109, which form a hydrophobic groove spanning EF-2 and -3, as well as R74,
K75, D78, E82, E83, R106, T110, and N111, which line the edges of this groove. Thus, as seen for Ca²⁺-ligated CaM
in complex with the same peptide (6), it is very likely that M13 binds as an amphipathic helix along this methionine-
rich cleft via hydrophobic contacts, augmented with elec-
trostatic and hydrogen bonding interactions to the flanking polar and charged side chains. Note that the M13 peptide is
grouped into the 1-5-8-14 class of CaM-binding sequences (43). Although we have not determined the 3D structure of
CaM/M-3 bound to M13, the general structural features of the unbound CaM/M-3 are most probably retained in the resulting
complex(es). Reasons for this include the presence of the hydrophobic binding groove in unbound CaM/M-3, the reten-
tion of the α-helices and bridging β-sheet between the EF-
hands upon complex formation, and the observation that the
structures of the N- and C-domains of Ca²⁺-ligated CaM do
not change significantly in the presence of target peptides
(1, 6). One notable exception, however, is that residues
75–79 adopt a new small helical segment in CaM/M-3/M13
as evident from their 13Cα-13Cβ secondary chemical shifts.
In free CaM/M-3, these residues form a flexible loop between EF-2 and -3, whereas in native CaM, they are part of the
interdomain linker. This linker exists as a long helix in the crystal structure of CaM (44–46), and in solution, it is
clearly conformationally dynamic (47), thereby facilitating
the formation of diverse target peptide complexes (1). In
these complexes, the linker residues generally do not
assume any regular secondary structure. However, in a recent
X-ray crystallographic analysis of CaM with a peptide
fragment corresponding to the CaM-binding site of cal-
cineurin, the linker was found to be helical (48). In the
case of CaM/M-3, M13 binding may lead to a small change in the relative positions of EF-2 and -3 that now accom-
domates the formation of a helix by the intervening linker residues.

Interaction of CaM/M-3 with Calcineurin. In addition to
binding the M13 peptide, CaM/M-3 has a limited ability to stimulate calcineurin and to inhibit the stimulation of this
phosphatase by CaM. In contrast, previous studies with the
N- and C-terminal tryptic fragments of CaM have shown
that neither of these fragments can influence the activity of
calcineurin (8).

Similar to the M13 peptide, calcineurin contains a 1-5-8-
14 CaM recognition sequence (1, 43). Thus, CaM/M-3 may
directly bind to the CaM-target site of calcineurin and thereby
stimulate its phosphatase activity. However, this effect is
significantly less than that observed with CaM, suggesting
that CaM/M-3 may not efficiently displace the auto-inhibitory
domain from the active site of calcineurin because of either
reduced binding affinity for or altered structural interactions
with its adjacent recognition sequence. Consistent with this
hypothesis, CaM/M-3 also inhibits the stimulation of cal-
cineurin by CaM. However, as shown in Figure 8B the
maximum initial rate of CaM stimulated phosphatase activity
of calcineurin decreases with increasing CaM/M-3, suggesting
an apparent noncompetitive, rather than competitive, mecha-


nism of inhibition. Unfortunately, we were unable to
investigate this further by carrying out the calcineurin
stimulation assays over a wider range of conditions because
high CaM concentrations (>300 nM) alone inhibit the
activity of calcineurin. Thus, the mechanism of inhibition
and the location of the binding site for CaM/M-3 on calcineurin
remain to be established.

Diversity of Target Recognition by CaM. CaM/M-3 involves
the non-native association of EF-2 and -3, and as such is
neither optimized for folding or specific target recognition.
Despite this, CaM/M-3 adopts a Ca²⁺-dependent structure
with peptide binding properties strikingly similar to those of the
N- or C-domains of intact CaM. This clearly exemplifies
the remarkable plasticity of EF-hand sequences to associate
into EF-hand domains and mediate Ca²⁺-dependent recogni-
tion of target proteins. Following the first structural descrip-
tion of CaM complexed with M13 (6), an amazing repertoire
of possible binding mechanisms have been discovered. These
include parallel and antiparallel orientations of target peptides
with respect to CaM (49) as well as varying stoichiometries
such as 1:1 (6, 50), 1:2 (51), and 2:2 (52) CaM/target-
peptide complexes. Examples have also been described where
a only single domain of CaM binds to a target (53) and where
target binding occurs in the presence and absence of Ca²⁺
(54). Apo-CaM has even been found as a domain-swapped
dimer (54). Expanding upon this diversity, CaM/M-3/M13 is
unusual in existing as an equilibrium between a 1:1 het-
erodimer and an asymmetric 2:2 heterotetramer. Understand-
ing the exact basis for this unusual asymmetry, which likely results from CaM/M-3 and M13 adopting two different,
spectroscopically distinct conformations at their binding
interfaces, awaits further characterization of related EF-hand
domains with additional target sequences.

REFERENCES

allows for promiscuity in its interactions with target proteins and


Regulation (Cohen, P., Ed.) pp 35–56, Elsevier Science
Publishers, Amsterdam.

conformational transition revealed by the solution structure of apo

5. Kuboniwa, H., Tjandra, N., Grzesiek, S., Ren, H., Klee, C. B.,
and Bax, A. (1995) Solution structure of calcium-free calmodulin,


terminal peptides of plant glutamate decarboxylase to calmodulin, 
*J. Mol. Biol.* 328, 193–204.


