Published in final edited form as:

J Am Chem Soc. 2020 December 16; 142(50): 21220–21232. doi:10.1021/jacs.0c11156.

Millisecond time-resolved solid-state NMR reveals a two-stage molecular mechanism for formation of complexes between calmodulin and a target peptide from myosin light chain kinase

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Abstract

Calmodulin (CaM) mediates a wide range of biological responses to changes in intracellular Ca^{2+} concentrations through its calcium-dependent binding affinities to numerous target proteins. Binding of two Ca^{2+} ions to each of the two four-helix-bundle domains of CaM results in major conformational changes that create a potential binding site for the CaM binding domain of a target protein, which also undergoes major conformational changes to form the complex with CaM. Details of the molecular mechanism of complex formation are not well established, despite numerous structural, spectroscopic, thermodynamic, and kinetic studies. Here we report a study of the process by which the 26-residue peptide M13, which represents the CaM binding domain of skeletal muscle myosin light chain kinase, forms a complex with CaM in the presence of excess Ca^{2+} on the millisecond time scale. Our experiments use a combination of selective ^{13}C labeling of CaM and M13, rapid mixing of CaM solutions with M13/ Ca^{2+} solutions, rapid freeze-quenching of the mixed solutions, and low-temperature solid state nuclear magnetic resonance (ssNMR) enhanced by dynamic nuclear polarization. From measurements of the dependence of 2D ^{13}C - ^{13}C ssNMR spectra on the time between mixing and freezing, we find that the N-terminal portion of M13 converts from a conformationally disordered state to an α -helix and develops contacts with the C-terminal domain of CaM in about 2 ms. The C-terminal portion of M13 becomes α -helical and develops contacts with the N-terminal domain of CaM more slowly, in about 8 ms. The level of structural order in the CaM/M13/ Ca^{2+} complexes, indicated by ^{13}C ssNMR linewidths, continues to increase beyond 27 ms.

Introduction

The 149-residue calcium-binding protein calmodulin (CaM) is found in all eukaryotes, with 100% sequence conservation in mammals, birds, reptiles, fish, and rays, and 59% conservation between *H. sapiens* and *S. cerevisiae*. CaM mediates the calcium sensitivity

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Supporting Information

Four additional figures, showing amino acid sequences and LC-MS data for M13 and CaM (Fig. S1), enhancements of ssNMR signals by DNP (Fig. S2), the dependences of intermolecular crosspeak volumes in 2D ^{13}C - ^{13}C ssNMR spectra of frozen CaM/M13/ Ca^{2+} solutions on the mixing time τ_{SD} (Fig. S3), and the dependences of intermolecular crosspeak volumes on the evolution time τ_{e} with $\tau_{\text{SD}} = 0.5$ s (Fig. S4).

of numerous cellular and physiological functions, including muscle contraction, long-term memory, cell cycle progression, and ion channel activity.¹⁻⁴ Upon binding of four Ca^{2+} ions to the two EF hand motifs in each of CaM's N-terminal and C-terminal domains, the apo-CaM conformation⁵ is converted to the holo-CaM conformation,⁶⁻⁷ which is capable of forming a hydrophobic channel that encloses the CaM-binding domain of a target protein (Fig. 1A).⁸⁻¹¹ Hundreds of target proteins for CaM have been identified. In some cases, the CaM-binding domain has an auto-inhibitory effect on the target protein until the CaM-binding domain binds to CaM. Interaction with CaM then relieves auto-inhibition, thereby activating the target protein, which can be a kinase, phosphatase, or other enzyme.¹²⁻¹⁴ Typically, the CaM-binding domain is natively unstructured but becomes α -helical within the hydrophobic channel of holo-CaM. Formation of a complex between CaM and a target protein following an increase in the intracellular Ca^{2+} concentration therefore involves major conformational changes in both protein components, possibly in a series of distinct stages.

Because of its central role in biology, calmodulin has been the subject of numerous studies, including numerous structural studies of various conformational states by nuclear magnetic resonance (NMR)^{5,7-8} and crystallography,^{6,9-10} as well as studies of molecular motions and conformational distributions at equilibrium in the presence and absence of calcium and target proteins or peptides.^{10,15-26} In addition to structural and dynamical studies at equilibrium, the four binding constants for Ca^{2+} have been characterized by various methods,²⁷⁻³⁰ binding constants for target proteins and peptides have been measured,^{28-29,31} and kinetics of Ca^{2+} and target protein binding have been studied by time-resolved optical spectroscopies,³¹⁻³⁶ mass spectrometry,²⁸ small angle x-ray scattering (SAXS),³⁷ and other methods.³⁸

Although previous studies provide important information about the process of CaM complex formation, existing information is incomplete. In particular, it has been difficult to obtain information about molecular-level details of the nonequilibrium, unidirectional structural changes that follow a rapid increase in Ca^{2+} concentration, because techniques such as optical spectroscopy and SAXS do not have high structural resolution. Moreover, the structural changes of interest occur quickly, on time scales of milliseconds. Standard high-resolution structural techniques, such as standard NMR and crystallography techniques, do not have the necessary time resolution when applied to nonequilibrium processes.

Recently, we introduced time-resolved solid state NMR (ssNMR) spectroscopy as a method for characterizing unidirectional structural conversion processes in biomolecular systems with site-specific spatial and millisecond time resolutions.³⁹ Our approach to time-resolved ssNMR uses a rapid mixing and freeze-quenching apparatus to initiate the structural conversion process and trap transient states that develop subsequently (Fig. 1B), with a minimum evolution time τ_e of 1-2 ms that is determined by the mixer volume, pumping rate, and minimal flight distance d_f . Rapidly frozen solutions with various isotopic labeling patterns and various values of τ_e can then be probed at leisure with a variety of ssNMR techniques to obtain information about the time dependence of conformations at specific residues, as well as the time dependence of intramolecular and intermolecular contacts (provided that the solutions are kept below their glass transition temperatures until measurements are completed). By performing ssNMR measurements at 25-30 K and using

dynamic nuclear polarization (DNP),⁴⁰⁻⁴³ we enhance the sensitivity of these measurements to the point where high-quality ¹³C-detected ssNMR data can be obtained in 1-10 h with protein concentrations below 1 mM and volumes below 100 μ l, even when the ssNMR spectra contain broad lines due to a high level of conformational disorder in transient structural states. Recent studies of the folding and self-assembly of melittin following a rapid pH jump demonstrate the ability of time-resolved ssNMR to provide new insights into the molecular-level details of a rapid biomolecular structural conversion process.³⁹

In experiments described below, we use time-resolved ssNMR spectroscopy to investigate the process of complex formation when a CaM solution is rapidly mixed with a solution containing Ca²⁺ and the 26-residue peptide M13,¹² which represents the CaM binding domain of skeletal muscle myosin light chain kinase (skMLCK).⁴⁴ M13 is unstructured in its unbound state and binds to CaM in the presence of Ca²⁺ with a binding constant of approximately 1 nM.⁴⁵ In the fully formed CaM/M13/Ca²⁺ complex, M13 becomes α -helical, with numerous contacts between the N-terminal portion of M13 and the C-terminal domain of CaM and between the C-terminal portion of M13 and N-terminal domain of CaM.^{8,11} Methionine sidechains from both domains of CaM line the hydrophobic channel that encloses M13, along with sidechains of Phe, Leu, Val, and Ala residues. Data presented below provide evidence that formation of this complex proceeds preferentially in two stages. Within about 2 ms, the N-terminal portion of M13 becomes helical and forms contacts with Met residues of the C-terminal domain of CaM. Subsequently, in about 8 ms, the C-terminal portion of M13 becomes helical and forms contacts with Met residues of the N-terminal domain of CaM. Although related two-stage processes have been proposed previously,^{31,36-37} the time-resolved ssNMR data provide a level of structural detail that has not been achieved with other experimental approaches.

Methods

Production of calmodulin and M13

CaM with the human sequence was expressed in BL21(DE3) *E. coli* cells using a pET15b expression vector. The cells were grown in defined amino acid medium (DAM) containing all amino acids, in addition to the conventional M9 minimal medium components. Methionine was ¹³C-labeled at the methyl position (¹³C_ε-Met-CaM). All other amino acids were unlabeled. Expression was induced at OD₆₀₀ $\approx$ 0.4 with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Cells were harvested after 4 h of incubation at 37° C with 250 rpm shaking. The cells were lysed in the lysis buffer containing 50 mM Tris-HCl, pH 7.5 with 2 mM ethylene diamine tetraacetic acid (EDTA) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The lysed cells were sonicated in an ice bath for 15 min at 0.5 output, 20% duty cycle, using a Branson Model 250 sonifier with a 1/8" tapered horn. Soluble protein was separated from cell debris by centrifugation at 141,000 $\times$ g at 4° C for 1 h. After adding 5 mM CaCl₂ to the supernatant, the protein was purified by using a hydrophobic interaction chromatography (HIC) Phenyl Sepharose column (GE Healthcare) with the Ca²⁺-containing loading buffer. The elution buffer was 50 mM Tris-HCl, pH 7.5, containing 1 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). Fractions containing calmodulin were dialyzed against deionized water for 24 h

with a 10 kDa cut-off dialysis membrane to remove residual Ca^{2+} and EGTA and produce the apo-CaM protein. After dialysis, calmodulin solutions were lyophilized.

An M13 peptide with ^{15}N , ^{13}C -labeled Lys5, Val11, Ala13, and Phe17 (M13-KVAF) was synthesized at 0.1 mmol scale on a Biotage Initiator+ Alstra solid-phase peptide synthesizer, using fluorenylmethoxycarbonyl (Fmoc) chemistry with diisopropylcarbodiimide/OxymaPure activation, and an H-Leu-HMPB-ChemMatrix resin (Sigma-Aldrich). The resin substitution was determined to be 0.4 meq/g, based on ultraviolet absorbance of the dibenzofulvene-piperidine adduct.⁴⁶ Most amino acids were single-coupled, with a five-fold excess of Fmoc-amino acid in N-methyl-2-pyrrolidone for 10 min at 75° C. Fmoc-Arg(Pbf)-OH and Fmoc-Trp(Boc)-OH were double-coupled at 75° C for 15 min. Isotopically labeled amino acids were coupled with a threefold excess for 20 min at 75° C, followed by coupling with a five-fold excess of the corresponding unlabeled amino acids for 5 min at 75° C. A single deprotection step with 20% piperidine in N,N-dimethylformamide with 0.1 M 1-hydroxybenzotriazole for 5 min at 70° C was used. Capping with acetic anhydride/N,N-diisopropylethylamine was performed before deprotection. The peptide was cleaved from the resin with a standard trifluoroacetic acid/phenol/thioanisole/1,2-ethanedithiol/water cocktail for 2.0 h. The crude product was purified by high-performance liquid chromatography on a Zorbax 300SB-C3 reverse-phase column (Agilent), using a water/acetonitrile gradient with 0.1% trifluoroacetic acid. An M13 peptide with ^{15}N , ^{13}C -labeled Phe8 and Ile20 and $^{13}\text{C}_\alpha$ -labeled Ala13 (M13-FAI) was purchased from Anaspec, Inc. during a period when the COVID-19 pandemic prevented us from synthesizing this material in our own laboratory.

Amino acid sequences of CaM and M13 are given in Fig. S1 of Supporting Information, along with liquid chromatography-mass spectrometry (LC-MS) data for purified $^{13}\text{C}_\text{e}$ -Met-CaM (expected MW = 16715.3 Da, observed MW = 16710.2) and M13-KVAF (expected MW = 2992.5 Da, observed = 2991.9 Da). These data were obtained with an Ultimate 3000 high-performance liquid chromatography system and MSQ Plus single-quadrupole mass spectrometer, using electrospray ionization, a water/acetonitrile gradient with 0.01% v/v trifluoroacetic acid, and an Acclaim PepMap 300 C18 reverse-phase column (Thermo Fisher Scientific).

Rapid mixing and rapid freezing

Our method for rapid mixing and freezing is depicted schematically in Fig. 1B. Details of the apparatus were described previously.³⁹ For rapid mixing experiments, a 100 μl volume of 3.0 mM M13 peptide and 40 mM CaCl_2 in 20 mM Tris-HCl, pH 7.0, was mixed with a 100 μl volume of 4.0 mM calmodulin in 20 mM Tris-HCl, pH 7.0, producing a final solution with 1.5 mM M13, 2 mM calmodulin, and 20 mM CaCl_2 in 20 mM Tris-HCl, pH 7.0. Both solutions contained 20% v/v perdeuterated, ^{13}C -depleted glycerol (Cambridge Isotope Laboratories) and 10 mM succinyl-DOTOPA.⁴⁷ The glycerol content was sufficient to produce homogeneous, glassy frozen solutions at the high cooling rates of our experiments, as demonstrated previously.³⁹ All samples also used a 1:7 ratio of H_2O to D_2O in the buffer.

The home-built mixer³⁹ is comprised of a Y-junction (Valco Instruments Co., part number C-NYXFPK) with 100 μm inner diameter (ID) channels, a 4 mm section of 100 μm ID

PEEKsil tubing packed with 40 μm stainless steel beads (Cospheric LLC, part number SSMMS-7.8) and capped with 10 μm mesh disks (Valco Instruments Co., part number 10SR.5-10) to contain the beads, and a nozzle comprised of 50 μm ID PEEKSil tubing. The mixer is contained in a polytetrafluoroethylene (PTFE) chamber maintained at 25° C and mounted on a stepper motor driven arm that sweeps across the surface of a rotating copper plate (18 cm diameter, 0.5 cm thickness, 300 rpm). The copper plate was cooled to 77 K in liquid nitrogen immediately before each sample preparation. The entire apparatus was enclosed in a home-built glove box, flushed with nitrogen gas, to prevent condensation of atmospheric moisture onto the copper plate.

The two sample solutions described above were loaded into two 22 cm lengths of 760 μm ID PEEK tubing and driven through the mixer by water from two high-pressure pumps (Scientific Systems, Inc., model LU005SFT1A). To separate the sample solutions from the driving water, 20 μl of hexane was loaded behind each sample solution. In-line filters (IDEX, part number M-543) were installed between the sample lines and the mixer to remove adventitious solid particles that might clog the mixer. As reported previously,³⁹ this setup allows us to mix the two sample solutions in about 1.6 ms at a total flow rate of 2.0 ml/min. The high-speed jet of mixed solution produced by the mixer freezes in less than 1 ms upon striking the cold copper surface.

We define the evolution time τ_e for each CaM/M13/Ca²⁺ sample to be the time required for the sample to pass through the mixer (0.053 μl internal volume without beads) and to fly the vertical distance d_f from the mixer nozzle to the copper plate. For $\tau_e = 1.5$ ms, the flow rate was 3.0 ml/min and $d_f = 1.0$ cm. For $\tau_e = 2.2$ ms and 8.2 ms, the flow rate was 2.0 ml/min and d_f was 1.0 cm and 11 cm, respectively. For $\tau_e = 27$ ms, a 30 mm length of 100 μm ID PEEKSil tubing was inserted before the 50 μm ID nozzle to increase the mixer volume to 0.25 μl . The flow rate and d_f were then set to 1.0 ml/min and 10 cm, respectively.

After freezing the rapidly mixed solutions on the copper plate, rotation of the plate was stopped. The frozen solution was scraped from the copper surface and packed into a magic-angle spinning (MAS) rotor (4.0 mm outer diameter, 80 μl sample volume⁴⁰), which had been placed in a funnel-shaped hole in the center of the copper plate. This packing procedure was performed entirely under liquid nitrogen to prevent warming of the sample. MAS rotors were stored in liquid nitrogen until the time of ssNMR measurements.

DNP-enhanced ssNMR

DNP-enhanced ssNMR measurements were performed at 9.4 T (100.8 MHz ¹³C NMR frequency) with a Bruker Avance III spectrometer console. An extended interaction oscillator (Communications & Power Industries) and quasi-optical interferometer (Thomas Keating Ltd.) provided 1.5 W of circularly polarized microwaves at 263.9 GHz.⁴⁰ The home-built DNP probe allows ssNMR measurements at 25-30 K sample temperatures and MAS frequencies up to at least 7.0 kHz, with a liquid helium consumption rate of 1.5 l/h.⁴⁰ For measurements on the rapidly frozen samples, the probe was pre-cooled to 25 K while spinning a dummy sample. Samples could then be changed manually by lowering the probe below the magnet, without detaching the liquid helium transfer line from the probe and without warming the samples above approximately 100 K.

For ^1H - ^{13}C cross-polarization, a ^1H radio-frequency (RF) field of 52 kHz and a ^{13}C RF field of 45 kHz were used at 7.00 kHz MAS frequency. A 51 kHz ^1H RF field and a 46 kHz ^{13}C RF field were used at 5.00 kHz MAS frequency. For ^1H decoupling, a ^1H RF field amplitude of 85 kHz was used with two-pulse phase modulation (TPPM).⁴⁸ 2D ^{13}C - ^{13}C spectra used spin diffusion mixing periods τ_{SD} . Maximum t_1 values were 8.0 ms, with t_1 increments of 40 μs for spectra with $\tau_{\text{SD}} = 20$ ms and 38 μs for spectra with $\tau_{\text{SD}} = 1.0$ s (to avoid overlap between intermolecular crosspeaks and aliased spinning sidebands). Recycle delays were set to $1.26\tau_{\text{DNP}}$, where τ_{DNP} was the build-up time for cross-polarized ^{13}C signals under microwave irradiation, obtained by fitting one-dimensional (1D) saturation-recovery data with single-exponential functions. Typically, $\tau_{\text{DNP}} = 3.5 \pm 0.5$ s. Cross-polarized ^{13}C signal enhancements ϵ from DNP (microwaves on vs. microwaves off) were typically about 100 for slowly frozen CaM/M13/ Ca^{2+} solutions containing 40% v/v glycerol and about 65 for rapidly frozen solutions containing 20% v/v glycerol (Table S1 and Fig. S2 of Supporting Information). With 1.5 mM M13 and 80 μl nominal sample volume (approximately 40 μl of granular frozen solution), 2D ^{13}C - ^{13}C spectra were acquired in 4-6 h. Double-quantum-filtered 1D ^{13}C spectra were acquired with SPC5 recoupling⁴⁹ for double-quantum excitation and mixing as previously described.³⁹

2D spectra were processed in NMRPipe⁵⁰ with 120-180 Hz Gaussian line broadening in both dimensions. Color-scale representations of 2D spectra in Figs. 2, 3, and 5 were created with Igor 6.37 (Wavemetrics, Inc.) from text files generated with the pipe2txt command of NMRPipe. Crosspeak volumes were measured in ImageJ as shown in Figs. 4 and 6, using tiff files generated with the pipe2tiff command of NMRPipe. Least-squares fitting of data in Figs. 4 and 6 was performed with Igor 6.37.

Results

Labeling strategy for monitoring M13 conformation and calmodulin/M13 contacts

As depicted in Fig. 1A, formation of the CaM-M13 complex in the presence of Ca^{2+} involves conversion of M13 from a conformationally disordered, unbound state to a bound state in which residues 5-20 adopt an α -helical conformation. In the final, fully structured complex, the N-terminal domain of CaM (residues 1-80, CaM-NTD) and the C-terminal domain of CaM (residues 81-148, CaM-CTD) form a cluster of α -helices with a central channel that accommodates the α -helical segment of M13. Each domain of CaM contains four α -helices and two Ca^{2+} -binding sites, with the Ca^{2+} ions being coordinated by carboxylate groups of Asp sidechains in loops between α -helical segments of CaM (*i.e.*, EF hand motifs). CaM-NTD makes contacts with amino acid sidechains in the C-terminal portion of the M13 α -helix (residues 9-11, 13, 14, and 16-18). CaM-CTD makes contacts with sidechains in the N-terminal portion of the M13 α -helix (residues 5, 7, 8, 12, and 15). CaM contains nine Met residues, an unusually high percentage (6.1%, compared with about 2% in all globular proteins⁵¹). The long hydrophobic sidechains of Met residues facilitate interactions with multiple residues of M13 peptide in the final complex,⁵² including Trp4, Phe8, Ala13, Ala14, and Phe17.

As shown schematically in Fig. 1B, solutions of apo-CaM (4.0 mM) were mixed rapidly with solutions of M13 (3.0 mM) and Ca^{2+} (40 mM) by pumping through a mixer, producing

final concentrations of 2.0 mM, 1.5 mM, and 20 mM, respectively. Mixed solutions were then frozen rapidly on a rotating, cold copper plate after an evolution time τ_e , determined by the distance between the mixer and the copper plate and by the pumping rate. Solutions contained 20% glycerol by volume, 20 mM Tris buffer (pH 7), and 10 mM succinyl-DOTOPA triradical. Frozen solutions were packed into 4.0 mm MAS rotors for ssNMR measurements, which were performed in a 9.4 T field at 25 K, with microwave irradiation for signal enhancement by cross-effect DNP. Other experimental details are given above.

For experiments described below, we introduced ^{13}C labels at all Met methyl sites in calmodulin. We used two M13 labeling patterns, the first being uniform ^{13}C labeling of Lys5, Val11, Ala13, and Phe17 of M13 (M13-KVAF) and the second being uniform ^{13}C -labeling of Phe8 and Ile20 and $^{13}\text{C}_\alpha$ -labeling of Ala13 (M13-FAI). This labeling strategy allowed us to monitor the time-dependence of the M13 conformation with residue-specific resolution across the helix-forming segment by recording a series of 2D ^{13}C - ^{13}C spectra with 20 ms periods for intra-residue polarization transfers by ^{13}C - ^{13}C spin diffusion (*i.e.*, $\tau_{\text{SD}} = 20$ ms). We could also monitor the development of intermolecular contacts between CaM Met sidechains and specific residues of M13 by recording 2D ^{13}C - ^{13}C spectra with larger τ_{SD} values. In the final, fully structured complex, the shortest M13/CaM carbon-carbon distances for Phe8/Met144, Phe8/Met145, Ala13/Met71, Ala13/Met72, Phe17/Met51, and Phe17/Met71 pairs are 9.7 Å, 3.5 Å, 3.8 Å, 5.4 Å, 3.4 Å, and 4.9 Å, respectively, based on the structure for the complex reported by Grishaev *et al.*¹¹ (Protein Data Bank code 2LV6). These ^{13}C - ^{13}C distances are within the range that can produce intermolecular ^{13}C - ^{13}C crosspeaks via homonuclear dipole-dipole interactions with $\tau_{\text{SD}} \sim 1$ s.

Fig. 1C shows a series of double-quantum-filtered one-dimensional ^{13}C ssNMR spectra of frozen CaM/M13-KVAF/ Ca^{2+} solutions with increasing values of τ_e . Double-quantum filtering suppresses both residual natural-abundance ^{13}C signals from glycerol and signals from the Met methyl labels in CaM. Spectra with $\tau_e = 0$ and $\tau_e = \infty$ correspond to a solution without CaM (*i.e.*, unbound M13) and a pre-mixed solution of all three components (*i.e.*, M13 in the fully formed complex). The progressive sharpening of spectral features and down-field shifts of C_α and CO lines with increasing τ_e in Fig. 1C indicate formation of the CaM/M13-KVAF/ Ca^{2+} complex on the experimentally accessible time scale.

It should be noted that values of τ_e in Fig. 1C, and in data discussed below, are nominal evolution times calculated from the mixer volume, the flow rate, and the flight distance d_f . In fact, the precise time point at which mixing occurs in our device varies by about ± 0.2 ms as solutions flow through the bead-packed section. The time to pass through the exit nozzle section varies by about 0.3 ms, due to the laminar flow profile. Therefore, each nominal value of τ_e represents a distribution of evolution times between mixing and freezing, covering approximately ± 0.4 ms. Spectra with a given τ_e value include contributions from CaM/M13/ Ca^{2+} complexes with evolution times that vary within this distribution.

Site-specific time-dependence of conformational order and secondary structure in M13

Fig. 2 shows contour plots of 2D ^{13}C - ^{13}C spectra with $\tau_{\text{SD}} = 20$ ms for frozen CaM/M13-KVAF/ Ca^{2+} solutions, obtained at MAS frequency $\nu_{\text{MAS}} = 7.00$ kHz. Spectra with $\tau_e = 0$ and $\tau_e = \infty$, as defined above, are shown in Fig. 2A. To facilitate visualization of crosspeak

shapes and intensities in the 2D spectra, we represent signal intensities with the color scales shown in the figures, rather than using the more traditional 2D contour plots. In Fig. 2A, color scales vary from purple to red for intensities between a minimum value λ that is slightly above the noise level of each spectrum and a maximum value equal to 50% of the $^{13}\text{C}_\alpha/^{13}\text{C}_\beta$ crosspeak height for Val11. Regions with intensities between 50% and 105% of the Val11 $^{13}\text{C}_\alpha/^{13}\text{C}_\beta$ crosspeak height are constant red. Regions with intensities below λ or above 105% of the Val $^{13}\text{C}_\alpha/^{13}\text{C}_\beta$ crosspeak height are white. Crosspeak widths (full width at half maximum) are about 6 ppm at $\tau_e = 0$ and about 2 ppm at $\tau_e = \infty$, consistent with a largely disordered state for unbound M13 and a well-ordered state in the final CaM/M13-KVAF/ Ca^{2+} complex.

In the Fig. 2B, 2D spectra of frozen solutions with $\tau_e = 1.5$ ms, 2.2 ms, 8.2 ms, and 27 ms are shown in the left-hand column, with intensities on the same color scale as in Fig. 2A. The spectrum at $\tau_e = 1.5$ ms shows a mixture of broad and sharp crosspeak signals, attributable to conformationally disordered and ordered M13 molecules, respectively. With increasing τ_e , the broad signal components decrease. Careful inspection indicates that the broad component of the Phe17 $^{13}\text{C}_\alpha/^{13}\text{C}_\beta$ crosspeak decreases more slowly than the broad components of Lys5, Val11, and Ala13 crosspeaks, suggesting that the process of complex formation does not occur in a single step that affects all labeled residues equally.

To allow quantification of the broad crosspeak components, 2D difference spectra $S_{\text{diff}}(\tau_e)$ were calculated according to $S_{\text{diff}}(\tau_e) = S(\tau_e) - \eta(\tau_e)S_\infty$ where $S(\tau_e)$ is the 2D spectrum at τ_e , S_∞ is the 2D spectrum at $\tau_e = \infty$, and $\eta(\tau_e)$ is a scaling factor that was adjusted to cancel out spectral intensity at 68.4 ppm/34.3 ppm, the position of the Val11 $^{13}\text{C}_\alpha/^{13}\text{C}_\beta$ crosspeak in S_∞ . The 2D difference spectra are shown in the right-hand column of Fig. 2B. The goal of this subtraction procedure is to eliminate signals from structured, helical M13 molecules in $S(\tau_e)$. Remaining signals in the difference spectra are then attributed to unstructured M13. Note that the 2D spectrum at $\tau_e = 0$ has essentially zero intensity at 68.4 ppm/34.3 ppm, to that intensity at this position arises only from structured, helical molecules. Color scales in the right-hand column of Fig 2B were set in the same way as in the left-hand column and in Fig 2A, except that the colors vary from purple to red between λ and 25% of the Val11 $^{13}\text{C}_\alpha/^{13}\text{C}_\beta$ crosspeak height observed in $S(\tau_e)$. Regions with intensities between 25% and 45% of the Val11 $^{13}\text{C}_\alpha/^{13}\text{C}_\beta$ crosspeak height are constant red. The 2D difference spectra confirm that most of the Phe17 $^{13}\text{C}_\alpha/^{13}\text{C}_\beta$ crosspeak volume remains at $\tau_e = 1.5$ ms, while volumes of other $^{13}\text{C}_\alpha/^{13}\text{C}_\beta$ crosspeaks are reduced. These crosspeak volumes continue to decrease with increasing τ_e , approaching the noise level at $\tau_e = 27$ ms.

Fig. 3 shows similar plots of 2D ^{13}C - ^{13}C spectra and 2D difference spectra for frozen CaM/M13-FAI/ Ca^{2+} solutions, obtained with $\tau_{\text{SD}} = 20$ ms. In this case, $^{13}\text{C}_\alpha/^{13}\text{C}_\beta$ crosspeaks of Phe8 and Ile20 overlap, interfering with residue-specific quantification of crosspeak volumes. In 2D spectra at $\nu_{\text{MAS}} = 7.00$ kHz (Fig. 3A,C), $^{13}\text{C}_\alpha/^{13}\text{C}_{\gamma 2,8}$ crosspeaks of Ile20 are resolved and can be used for quantification. Crosspeaks between $^{13}\text{C}_\alpha$ and aromatic ^{13}C lines of Phe8 (*i.e.*, $^{13}\text{C}_\alpha/^{13}\text{C}_{\text{aromatic}}$ crosspeaks) could potentially be used for quantification, but overlap with spinning sideband signals at $\nu_{\text{MAS}} = 7.00$ kHz. Therefore, 2D ^{13}C - ^{13}C spectra were also obtained at $\nu_{\text{MAS}} = 5.00$ kHz (Fig. 3B,D). In Figs. 3A and 3B, down-field shifts of Phe8 and Ile20 $^{13}\text{C}_\alpha$ signals are evident, based on the $^{13}\text{C}_\alpha$ - $^{13}\text{C}_{\gamma 2,8}$

(Fig. 3A) and $^{13}\text{C}_\alpha$ - $^{13}\text{C}_{\text{aromatic}}$ (Fig. 3B) crosspeak positions. Fig. 3C and D show 2D spectra at $\tau_e = 1.5$ ms, 2.2 ms, 8.2 ms, and 27 ms, as well as 2D difference spectra calculated as described above. For the difference spectra in Fig. 3C, the scaling factor $\eta(\tau_e)$ was adjusted to cancel out spectral intensity at 65.4 ppm/18.5 ppm, the position of the Ile20 $^{13}\text{C}_\alpha$ - $^{13}\text{C}_{\gamma 2,6}$ crosspeak in S_∞ . For the difference spectra in Fig. 3D, $\eta(\tau_e)$ was adjusted to cancel out spectral intensity at 62.3 ppm/131.1 ppm, the position of the Phe8 $^{13}\text{C}_\alpha$ - $^{13}\text{C}_{\text{aromatic}}$ crosspeak in S_∞ . Again, this subtraction procedure is intended to eliminate signals from structured, helical M13 molecules in $S(\tau_e)$ for each value of τ_e , with remaining signals therefore arising from unstructured M13. In the 2D difference spectra, significant Ile20 signal volume remains at $\tau_e = 8.2$ ms, whereas signals from Phe8 disappear more quickly.

The precise values of $\eta(\tau_e)$ for difference spectra in Figs. 2 and 3 are listed in Table S1 of the Supporting Information. These values are affected by variations in sample volumes, signal averaging, and DNP signal enhancement factors. Therefore, the $\eta(\tau_e)$ values themselves do not have a simple interpretation.

For quantitative analysis of site-specific kinetics of structure formation, crosspeak volumes within regions shown in Figs. 4A-C were measured in both the original 2D spectra $S(\tau_e)$ and the difference spectra $S_{\text{diff}}(\tau_e)$. These regions were carefully defined to cover signals from both structured and unstructured M13 molecules at all τ_e . Peak volume ratios $R(\tau_e) = V_{\text{diff}}(\tau_e)/V(\tau_e)$ were then calculated, where $V(\tau_e)$ and $V_{\text{diff}}(\tau_e)$ are volumes in $S(\tau_e)$ and $S_{\text{diff}}(\tau_e)$. Fig. 4D shows plots of the quantity $1-R(\tau_e)$, *i.e.*, plots of the build-up of signals from conformationally ordered M13 molecules at the residue-specific level. Error bars in these plots were calculated from the root-mean-squared errors in crosspeak volumes due to noise in the 2D spectra, determined by measuring noise signal volumes in multiple regions of the 2D spectra that were free of real crosspeak signals. Residue-specific build-up times τ_{bu} were then determined by fitting the experimental data with single-exponential expressions $A - B \exp(-\tau_e/\tau_{\text{bu}})$. In agreement with the qualitative observations given above for Figs. 2 and 3, build-up times were significantly longer for Phe17 and Ile20 than for Lys5, Phe8, Val11, and Ala13 ($\tau_{\text{bu}} \approx 8$ ms vs. $\tau_{\text{bu}} \approx 3$ ms). Thus, it appears that the N-terminal portion of M13 becomes conformationally ordered significantly more rapidly than the C-terminal portion in the process of CaM/M13/ Ca^{2+} complex formation.

Time-dependence of intermolecular contacts involving Met sidechains of calmodulin

2D ^{13}C - ^{13}C spectra with larger values of τ_{SD} can contain "long-range" crosspeaks between ^{13}C -labeled sites with internuclear distances greater than 5 Å. With the labeling patterns described above, we expect to see crosspeaks between ^{13}C -labeled Met methyl sites of CaM and Phe8, Ala13, and Phe17 of M13 when the CaM/M13/ Ca^{2+} complexes are fully formed. The build-up of these crosspeaks with increasing τ_e could then provide information about the development of intermolecular structure, which we can correlate with the development of conformational order in M13 discussed in the previous section.

^{13}C NMR chemical shifts of the nine Met methyl sites of CaM occur in the 13-23 ppm range. As shown in Fig. 5, 2D ^{13}C - ^{13}C spectra of CaM/M13-KVAF/ Ca^{2+} and CaM/M13-FIA/ Ca^{2+} at $\tau_e = \infty$ with $\tau_{\text{SD}} = 1.0$ s do indeed show crosspeaks attributable to short

intermolecular distances between Met methyl sites and Phe17 of M13 (Fig. 5A), and between Met methyl sites and Phe8 and Ala13 of M13 (Fig. 5B). Color scales in these 2D spectra vary from purple to red between a minimum value λ that is slightly above the noise level of each spectrum and a maximum value equal to 50% of the Val11 $^{13}\text{C}_\alpha/^{13}\text{C}_\gamma$ or Phe8 $^{13}\text{C}_\alpha/^{13}\text{C}_\beta$ crosspeak height (Fig. 5A or Fig. 5B, respectively). Regions with intensities between 50% and 105% of these crosspeak heights are constant red.

Note that Ala13 in M13-FAI (Fig. 5B) was labeled only at the C_α position, so that crosspeaks to Met methyl sites of CaM could be observed without interference from intra-residue $^{13}\text{C}_\alpha/^{13}\text{C}_\beta$ crosspeaks of Ala13. In fact, the Ala13 $^{13}\text{C}_\alpha$ signal of M13 shows crosspeaks to two different Met methyl signals, at 19.5 ppm and 15.5 ppm, attributable to intermolecular distances of 4.3 Å and 5.4 Å to methyl carbons of Met71 and Met72 of CaM in the fully formed complex¹¹. The shortest distances from aromatic carbons of Phe17 to Met71 and Met72 methyl carbons of CaM are 4.9 Å and 9.9 Å, respectively. Since the Phe17 aromatic carbon signals show a strong intermolecular crosspeak to a methyl signal at 19.5 ppm in Fig. 5A, but no crosspeak intensity near 15.5 ppm, we assign the signals at 19.5 ppm and 15.5 ppm to Met71 and Met72, respectively. Since Phe17 also has a 3.4 Å distance to the methyl carbon of Met51, we can also assign a chemical shift of approximately 19.5 ppm to the Met51 methyl carbon, based on the reasonable expectation that crosspeaks from Phe17 to both Met51 and Met71 should be observable in 2D ^{13}C - ^{13}C spectra of the fully formed complex when $\tau_{\text{SD}} = 1.0$ s. Similarly, the shortest distances from aromatic carbons of Phe8 to Met methyl carbons of CaM are 3.2 Å to Met145 and 7.5 Å to Met72. The crosspeak from Phe8 aromatic signals to Met methyl signals occurs at 21.4 ppm in Fig. 5B. Therefore, we assign a chemical shift of 21 ppm to the methyl carbon of Met 145.

To compare residue-specific kinetics of the formation of intermolecular contacts, we measured crosspeak volumes within the regions shown in Figs. 6A and 6B. For Phe8 and Phe17, ratios of volumes of intermolecular crosspeaks between Phe aromatic and Met methyl signals (*i.e.*, $^{13}\text{C}_{\text{aromatic}}/^{13}\text{C}_{\text{Met}}$ crosspeaks) to volumes of intramolecular Phe $^{13}\text{C}_{\text{aromatic}}/^{13}\text{C}_\beta$ crosspeaks were evaluated and normalized to the volume ratios at $\tau_e = \infty$. For Ala13, ratios of the volume of intermolecular $^{13}\text{C}_\alpha/^{13}\text{C}_{\text{Met}}$ crosspeaks to the volume of the $^{13}\text{C}_\alpha/^{13}\text{C}_\alpha$ diagonal peak were used. The resulting build-up data are plotted in Fig. 6C, with error bars determined from the root-mean-squared noise volumes in regions of the 2D spectra that were free of real crosspeak signals. As shown in Fig. 6C, fits with single-exponential curves indicate a significantly more rapid build-up for Phe8 than for Ala13 and Phe17. Thus, intermolecular crosspeaks also indicate a more rapid development of structure at the N-terminal side of M13, relative to the C-terminal side. Since Phe8 has its one short distance to Met145, while Ala13 and Phe17 have short distances to Met49, Met71, and Met72, it appears that CaM-CTD makes structurally ordered contacts to the N-terminal portion of M13 before CaM-NTD makes structurally ordered contacts to the C-terminal portion of M13.

For both Phe8 and Phe17, build-up times for intermolecular contacts in Fig. 6C are the same to within experimental uncertainty as build-up times for conformational order in Fig. 4D. For Ala13, the build-up time for conformational order (2.7 ± 0.8 ms) is shorter than the build-up time for intermolecular contacts (9.0 ± 4.2 ms). Apparently, Ala13 adopts α -helical

structure when M13 binds to CaM-CTD, before forming structurally ordered contacts with CaM-NTD.

Dependence of intermolecular crosspeak volumes on the mixing period in 2D ^{13}C - ^{13}C spectra

For frozen CaM/M13-KVAF/ Ca^{2+} and CaM/M13-FAI/ Ca^{2+} solutions with $\tau_e = \infty$, ratios of intermolecular crosspeak volumes to intramolecular crosspeak (or diagonal peak) volumes were evaluated as described above for ^{13}C - ^{13}C mixing periods τ_{SD} between 20 ms and 1.0 s. Crosspeak volume ratios increase with characteristic spin diffusion times (not to be confused with the build-up times for structure formation discussed above) in the 400-800 ms range (Fig. S3 of Supporting Information). For Ala13, the characteristic spin diffusion time for its crosspeak to Met71 is somewhat shorter than the characteristic time for its crosspeak to Met72 (500 ms vs. 700 ms), consistent with a shorter distance to the Met71 methyl carbon.

At $\tau_e = \infty$ and $\tau_{\text{SD}} = 1.0$ s, the ratio of the intermolecular $^{13}\text{C}_{\text{aromatic}}/^{13}\text{C}_{\text{Met}}$ crosspeak volume to the intramolecular $^{13}\text{C}_{\text{aromatic}}/^{13}\text{C}_{\beta}$ crosspeak volume was 2.0 ± 0.1 for Phe17, consistent with contributions from both Met51 and Met71 and with full equilibration of ^{13}C spin polarizations for ^{13}C - ^{13}C distances less than 5 Å. The corresponding crosspeak volume ratio for Phe8 was approximately 1.1 ± 0.2 , consistent with a significant contribution only from polarization transfer to Met145. Since CaM contains nine Met residues, with nearest-neighbor inter-residue methyl-methyl distances in the 4-11 Å range, volumes of crosspeaks from M13 to CaM within fully formed complexes can continue to increase beyond the value of τ_{SD} at which ^{13}C spin polarizations are equilibrated within 5 Å radii.

For all frozen CaM/M13-FAI/ Ca^{2+} solutions, 2D ^{13}C - ^{13}C spectra were also recorded with $\tau_{\text{SD}} = 0.5$ s (Fig. S4A of Supporting Information). Analysis of crosspeak volumes (Fig. S4B of Supporting Information) resulted in the same values of residue-specific build-up times for intermolecular contacts as in Fig. 6C, within experimental uncertainty.

Discussion

Summary of experimental results

Qualitatively, the dependence of ^{13}C ssNMR signals on τ_e (Figs. 1C, 2, and 3) shows that M13 undergoes major conformational changes within 2 ms of mixing with CaM in the presence of Ca^{2+} . ^{13}CO and $^{13}\text{C}_{\alpha}$ signals move downfield (*i.e.*, to larger chemical shift values), indicating formation of α -helical secondary structure at all labeled residues (Table 1). However, the 1D and 2D spectra continue to change up to and beyond $\tau_e = 27$ ms, indicating a gradual reduction in conformational disorder. 2D difference spectra in Figs. 2 and 3, in which crosspeak signals from the fully formed CaM/M13/ Ca^{2+} complexes are subtracted from spectra at intermediate values of τ_e , allow us to quantify the remaining populations of conformationally disordered M13 molecules at a residue-specific level. This analysis leads to the conclusion that residues in the N-terminal portion of M13 (Lys 5, Phe8, Val11, Ala13) become conformationally ordered on the 2 ms time scale, while residues in the C-terminal portion (Phe17, I20) become conformationally ordered on the 8 ms time scale (Fig. 4D).

The two-stage mechanism for complex formation is supported further by measurements of intermolecular contacts, through crosspeaks between ^{13}C -labeled sites in M13 and ^{13}C -labeled Met methyl sites in CaM in 2D spectra with $\tau_{\text{SD}} = 1.0$ s (Fig. 5) and $\tau_{\text{SD}} = 0.5$ s (Fig. S3 of Supporting Information). With increasing τ_e , intermolecular crosspeaks involving Phe8 build up more rapidly than intermolecular crosspeaks involving Ala13 and Phe17, again with time scales of about 2 ms and 8 ms (Figs. 6 and S3). Met residues that participate in these intermolecular crosspeaks can be assigned, based on interatomic distances in the known structure of the fully formed CaM/M13/ Ca^{2+} complex¹¹. Since Phe8 of M13 has a 3.2 Å carbon-carbon distance to Met145 of CaM, Ala13 of M13 has 4.3 Å and 5.4 Å distances to Met71 and Met72 of CaM, and Phe17 of M13 has 3.4 Å and 4.9 Å distances to Met51 and Met71 of CaM, we conclude that CaM-CTD develops contacts with M13 in the first stage of complex formation, while CaM-NTD develops contacts with M13 in the second stage.

Thus, it appears that intermolecular contacts develop concomitantly with conformational order of M13, on a residue-specific level. It is worth noting that ^{13}C ssNMR linewidths of labeled sites in M13 continue to decrease on longer time scales, as is apparent from differences between spectra at $\tau_e = 27$ ms and $\tau_e = \infty$ (Figs. 1C, 2, and 3). These spectral changes suggest that structural order within CaM/M13/ Ca^{2+} complexes continues to increase after residues 5-20 of M13 become fully α -helical, perhaps through optimization or annealing of intra- and intermolecular sidechain packing throughout the complex.

As described in the Methods section, our solutions contain 20% v/v glycerol, or 24% by weight. At 25° C, the temperature at which rapid mixing of CaM with M13 and Ca^{2+} occurs, the viscosity of the solutions is approximately 1.8 cP.⁵⁴ Therefore, we expect the presence of glycerol to affect the time scales for structure formation discussed above by a factor less than two.⁵⁵ On the other hand, the viscosity increases by a factor of 2.4 as the temperature decreases to 0° C and by an additional factor of 2.8 as the temperature decreases to -25° C.⁵⁶ When the jet of mixed solution strikes the cold, rotating copper surface of our apparatus, the solution is expected to reach -25° C in less than 0.3 ms,⁵⁷⁻⁵⁸ since the thickness of the frozen solution on the copper surface is less than 50 μm . Any potential temperature-dependent structural changes during the freezing process would be impeded by the increasing viscosity of the solution.

Relation to previous studies

The four EF hands of CaM bind Ca^{2+} with different affinities. Average binding constants for the two EF hands in CaM-CTD are about 4 μM at physiological ionic strength, while average binding constants for the two EF hands in CaM-NTD are about 20 μM .²⁷⁻²⁹ With increasing concentration, Ca^{2+} binds to the four EF hands preferentially in order from the C-terminal end to the N-terminal end of CaM.³⁰ Although the greater affinity for Ca^{2+} in CaM-CTD does not necessarily indicate faster kinetics, these thermodynamic factors are consistent with the more rapid development of interactions between M13 and CaM-CTD indicated by our time-resolved ssNMR data.

Several sets of stopped-flow fluorescence measurements of bimolecular association rates for CaM and target proteins in the presence of excess Ca^{2+} have been reported. The association

rates range from approximately $5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for complexes of CaM with full-length skMLCK³³ or calcineurin³⁵ to $5 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ for complexes with peptides from the CaM binding domain of nitric oxide synthase.³⁴ Interferometry-based biosensor measurements indicate an association rate of approximately $1.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ for the complex of CaM with full-length nitric oxide synthase.³⁸ Extrapolation of the stopped-flow fluorescence results to our experimental conditions suggests that association between CaM and M13 would take less than 0.1 ms. Although it is conceivable that unstructured CaM/M13/Ca²⁺ complexes form on such short time scales, our data indicate that structure formation within such complexes is substantially slower. The apparent discrepancy with stopped-flow fluorescence measurements is attributable to the low protein concentrations in those measurements. At low concentrations, the rate-limiting step for changes in fluorescence intensity is the initial productive encounter between CaM and the target protein, rather than structural changes within the complex. Our time-resolved ssNMR experiments are performed in a regime where the initial encounter occurs very quickly compared with the subsequent structural evolution. Our experiments monitor the structural evolution within CaM/M13/Ca²⁺ complexes, rather than the kinetics of the initial encounter.

Park *et al.* reported a series of stopped-flow and microfluidic continuous flow fluorescence measurements on the kinetics of Ca²⁺ binding to CaM, in the absence of a target protein.³⁶ At high [Ca²⁺], they observed rapid binding of the first two Ca²⁺ ions, within about 0.5 ms, followed by slower binding of the second two Ca²⁺ ions, in about 20 ms. Their data suggest that the binding rate of the second two Ca²⁺ ions is limited by conformational changes within CaM-NTD, although the specific nature of these conformational changes is unclear. In light of these results, it seems possible that the 8 ms time scale for development of conformational order and intermolecular contacts in the C-terminal portion of M13 observed in our time-resolved ssNMR measurements may be determined in part by the rate of binding of Ca²⁺ to CaM-NTD. This Ca²⁺ binding rate may increase when a target peptide is already bound to CaM and partially structured.

Yamada *et al.* used time-resolved SAXS and a photo-labile Ca²⁺ chelator to study the formation of complexes of CaM with mastoparan, triggered by release of excess Ca²⁺.³⁷ The time-dependent radius of gyration R_g extracted from their SAXS data showed a process that occurred in about 3 ms, similar to the 2 ms time scale in our time-resolved ssNMR data, followed by a process that occurred in about 40 ms. Yamada *et al.* interpreted the faster process as the binding of two Ca²⁺ ions to CaM and the slower process as subsequent binding of mastoparan with two additional Ca²⁺ ions. This interpretation is based in part on the time-dependence of the forward scattering intensity $I(0)$, which indicates an increase in molecular mass during the slower phase. However, the change in $I(0)$ is relatively small and the experiments of Park *et al.* indicate that binding of the first two Ca²⁺ ions should occur on a sub-millisecond time scale.³⁶ It seems possible that mastoparan binds to CaM during the faster process in the SAXS data, which would be more consistent with our time-resolved ssNMR data. On the other hand, it is also possible that the mechanisms of formation of CaM/mastoparan/Ca²⁺ and CaM/M13/Ca²⁺ complexes are different, especially since mastoparan is only 14 residues in length and lacks the Trp and Phe residues that participate in interactions with CaM in complexes of CaM with M13 and other target peptides.¹²

In another related study, Murase and Io used stopped-flow fluorescence measurements to study the formation of complexes of CaM with mastoparan X.³¹ In the presence of excess Ca^{2+} , they observed rapid fluorescence changes, in 1-2 ms, followed by slower changes, in about 50 ms. Based in part on additional experiments with N-terminal and C-terminal fragments of CaM, they attributed the slow phase to conversion of an intermediate, in which mastoparan X is bound to CaM-NTD, to the fully formed complex. This interpretation apparently conflicts with our time-resolved ssNMR data, which show that interactions of CaM-CTD with a target peptide precede interactions of CaM-NTD. It should be noted that the fluorescence measurements do not provide direct information about the conformation of the target peptide or about the identities of interacting domains. Again, it is also possible that different target peptides form complexes with CaM by different molecular mechanisms.

Studies of the activation of skMLCK and other target proteins by N-terminal and C-terminal fragments of CaM in the presence of Ca^{2+} suggest that CaM-CTD binds to these target proteins with greater affinity than CaM-NTD.⁵⁹ It has been suggested that a partially structured complex, with the target protein bound to CaM-CTD but not yet activated, may exist within cells at intermediate intracellular Ca^{2+} concentrations, thereby facilitating rapid activation when the Ca^{2+} concentration rises further.^{29,60} Our time-resolved ssNMR measurements on the CaM/M13/ Ca^{2+} system show that partially structured complexes are a kinetic intermediate, complementing these earlier equilibrium studies.

Future directions

Having demonstrated the feasibility and information content of time-resolved ssNMR measurements on CaM complex formation, it will be interesting to extend these measurements in several directions. For example, experiments in which CaM/ Ca^{2+} solutions are mixed with M13 solutions can test whether the molecular mechanism for complex formation changes when the initial state includes holo-CaM rather than apo-CaM. Experiments with rare disease-associated CaM mutations, which occur primarily in CaM-CTD,⁶¹ can test whether a change in the relative Ca^{2+} binding affinities of CaM-CTD and CaM-NTD affects the molecular mechanism for complex formation. Experiments with other target peptide sequences can test the influence of specific intermolecular interactions on the molecular mechanism. Measurements of τ_e -dependent distance distributions with pulsed electron paramagnetic resonance techniques can provide complementary structural information.⁶² Extensions to other peptide/protein complexes are readily envisioned.

Conclusions

Time-resolved ssNMR data presented above provide new insights into the process of CaM/M13/ Ca^{2+} complex formation. The site-specificity of structural information from ssNMR and the sensitivity of ssNMR spectra to local structure and conformational order, together with the high time resolution of our rapid mixing and freeze-quenching apparatus, permit an unambiguous characterization of essential aspects of the molecular mechanism that have not been accessible in previous studies. Our data show that a partially structured complex forms in about 2 ms after mixing a CaM solution with a M13/ Ca^{2+} solution. Within this intermediate complex, the N-terminal portion of M13, including residues 5-13,

has a well-ordered α -helical conformation and makes contacts with methionine sidechains of CaM-CTD. More slowly, in about 8 ms, the C-terminal portion of M13 adopts a well-ordered α -helical conformation and makes contacts with CaM-NTD. The overall level of molecular structural order in the complex continues to evolve on a longer time scale, as evidenced by reductions in ^{13}C ssNMR linewidths in frozen CaM/M13/ Ca^{2+} solutions with $\tau_c > 8$ ms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. J.J. was supported by a Korean Visiting Scientist Training Award from Korea Health Industry Development Institute.

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Figure 1.

(A) Schematic representation of CaM/M13/Ca²⁺ complex formation. Residues of M13 that are isotopically labeled in time-resolved ssNMR experiments are shown in magenta. N-terminal and C-terminal domains of CaM are blue and beige. The apo-CaM structure is based on Protein Data Bank file 1CFC. The fully formed complex structure is based on Protein Data Bank file 2LV6, with four bound Ca²⁺ ions shown in green. (B) Schematic representation of the rapid mixing and freeze-quenching apparatus used to prepare frozen CaM/M13/Ca²⁺ solutions for time-resolved ssNMR measurements. The evolution time τ_e of a given sample is determined by the velocity of a jet of CaM/M13/Ca²⁺ solution from the mixer and by the flight distance d_f before freezing. (C) Double-quantum-filtered ¹³C ssNMR spectra of frozen solutions with the indicated τ_e values. Spectra were obtained at 25 K and 9.4 T, with 7.00 kHz MAS and signal enhancements from DNP.

Figure 2.

(A) 2D ^{13}C - ^{13}C ssNMR spectra of a frozen M13-KVAF/ Ca^{2+} solution ($\tau_e = 0$) and a frozen, premixed CaM/M13-KVAF/ Ca^{2+} solution ($\tau_e = \infty$). Color scales for crosspeak intensities are explained in the text. $^{13}\text{C}_\alpha/^{13}\text{C}_\beta$ crosspeak assignments for labeled residues in M13 at $\tau_e = \infty$ are shown in orange. Dashed grey lines illustrate ^{13}C chemical shift changes that accompany the conversion of M13 from a conformationally disordered unbound state to an α -helical CaM-bound state. Spectra were acquired with 7.00 kHz MAS and ^{13}C - ^{13}C mixing periods $\tau_{\text{SD}} = 20$ ms. (B) 2D ^{13}C - ^{13}C ssNMR spectra of frozen M13-KVAF/ Ca^{2+} solutions with the indicated values of τ_e (left column) and 2D difference spectra (right column), in which the spectrum at $\tau_e = \infty$ is subtracted from spectra at the indicated values of τ_e as explained in the text. Crosspeak signals in the difference spectra are attributed to disordered components of the conformational distribution of M13 at each value of τ_e . The notation "(x ms)-(∞)" represents the difference between 2D spectra with $\tau_e = x$ and $\tau_e = \infty$, including the scaling factor η defined in the text.

Figure 3:

Same as Fig. 2, but for frozen CaM/M13-FAI/Ca²⁺ solutions. 2D ¹³C-¹³C ssNMR spectra were recorded with MAS at 7.00 kHz (A,C) and 5.00 kHz (B,D).

Figure 4: (A,B,C) 2D ^{13}C - ^{13}C ssNMR spectra of frozen solutions of CaM/M13-KVAF/ Ca^{2+} , CaM/M13-FAI/ Ca^{2+} at 7.00 kHz MAS frequency, and CaM/M13-FAI/ Ca^{2+} at 5.00 kHz MAS frequency, respectively, with $\tau_e = \infty$ and $\tau_{SD} = 20$ ms. Regions that are used to evaluate crosspeak volumes are indicated. (D) Residue-specific build-up curves for crosspeak signals attributable to conformationally ordered M13 molecules, determined from ratios of crosspeak volumes in the 2D spectra and 2D difference spectra in Figs. 2 and 3. Build-up times τ_{bu} were determined from single-exponential fits, shown as dashed lines.

Figure 5:

(A,B) 2D ^{13}C - ^{13}C ssNMR spectra of frozen CaM/M13-KVAF/ Ca^{2+} solutions and frozen CaM/M13-FAI/ Ca^{2+} solutions, respectively acquired with $\tau_{\text{SD}} = 1.0$ s. Intermolecular crosspeaks between ^{13}C -labeled sites in M13 and ^{13}C -labeled methionine methyl carbons of CaM are indicated in orange.

Figure 6:

(A,B) 2D ^{13}C - ^{13}C ssNMR spectra of frozen solutions of CaM/M13-KVAF/ Ca^{2+} and CaM/M13-FAI/ Ca^{2+} , respectively, with $\tau_e = \infty$ and $\tau_{SD} = 1.0$ s. Regions that are used to evaluate crosspeak volumes are indicated. (C) Residue-specific build-up curves for intermolecular contacts between M13 and methionine sidechains of CaM, determined from ratios of intermolecular and intramolecular crosspeak volumes in the 2D spectra in Fig. 5. Build-up times τ_{bu} were determined from single-exponential fits, shown as dashed lines.

Table 1:

^{13}C NMR chemical shifts (ppm) for labeled residues in the M13 peptide in frozen solutions of the fully formed CaM/M13/ Ca^{2+} complex. Chemical shifts are referenced to the ^{13}C line of glycerol in the frozen solution, taken to be 74.8 ppm relative to DSS. Uncertainties are approximately ± 0.2 ppm. Values in parentheses are random coil shifts reported by Wishart *et al.*⁵³

M13 residue	CO	C $_{\alpha}$	C $_{\beta}$	C $_{\gamma}$ C $_{\delta}$	Met C $_{\epsilon}$ of CaM within 6 Å
Lys5	180.0 (176.6)	58.1 (56.2)	28.5 (33.1)		
Phe8	180.8 (175.8)	62.3 (57.7)	42.6 (39.6)		21.4 (Met145)
Val11	179.7 (176.3)	68.4 (62.2)	34.3 (32.9)	24.0	
Ala13	180.0 (177.8)	57.3 (52.5)	20.7 (19.1)		19.5 (Met71), 15.5 (Met72)
Phe17	180.3 (175.8)	63.2 (57.7)	39.7 (39.6)		19.5 (Met51, Met71)
Ile20	177.8 (176.4)	65.4 (61.1)	40.6 (38.8)	31.7, 18.5	