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Supplemental Information

Structural Basis for Calmodulin as a Dynamic Calcium Sensor

Miao Zhang, Cameron Abrams, Liping Wang, Anthony Gizzi, Liping He, Ruihe Lin, Yuan Chen, Patrick J. Loll, John M. Pascal, and Ji-fang Zhang

Inventory of Supplemental Information

(1) supplemental results, which include six figures related to Main Figures 1, 3, 4, 5, 6 and 7; and two tables, one is related to main Figure 3 (parameters derived from ITC data), the other shows details of results of molecular modeling.

(2) Supplemental Experimental Procedures.

Supplemental Information

Supplemental Figure Legends

Figure S1 (related to Figure 1). Final electron density maps for the structure of the CaM-CaMBD2-b complex and comparison of the structure of CaM-CaMBD2-b with that of 1K90 or 1G4Y.

(A – D) 2Fo-Fc map was calculated using weighted coefficients output by the program PHENIX. The map is contoured at the level of 1σ. The refined model has been superimposed on the electron density map in the following regions of the structure: (A) overall view of the complex, (B) section of the hydrophobic core of the CaM N-lobe, (C) detailed view of a Ca^{2+} binding site in the CaM C-lobe, and (D) region of the CaMBD2-b fragment bearing the ARK insert.

(E) Superimposition of the structures of CaM from the CaM-CaMBD2-b complex (salmon) and the complex of CaM and edema factor (pale cyan, 1K90). The CaM structures are aligned at the C-lobe. Even though CaM in 1K90 is considered in the extended mode, its linker region still unwinds.

(F & G) An open-mode configuration of the CaM C-lobe in both CaM-CaMBD2-b and CaM-CaMBD2-a complexes. (F) The CaM C-lobe from CaM-CaMBD2-b (salmon) is superimposed on that from CaM-CaMBD2-a (blue). (G) The CaM C-lobe from CaM-CaMBD2-b (salmon) is superimposed on that from the CaM edema factor complex (pale cyan).

Figure S2 (related to Figure 3). Thermodynamic profiles of CaM-CaMBD2-b and CaM-CaMBD2-a.

Ca^{2+}-bound CaM was titrated into CaMBD2-b (A) or CaMBD2-a (B) in the presence of Ca^{2+} at 20°C. Smooth curves are fitting of the ITC data to the one-site model (A) and the two-site model (B)

Figure S3 (related to Figure 4). Structural details of the hydrophobic interface the CaM N-lobe in CaM-CaMBD2-b and CaM-CaMBD2-a.

The CaM N-lobe has the near identical conformation in both CaM-CaMBD2-b (salmon) and CaM-CaMBD2-a (blue). Superimposition of the binding interfaces between CaM N-lobe and CaMBDs. Both structures are aligned at the C-terminal region of the CaMBD2-b and CaMBD2-a peptides.

Figure S4 (related to Figure 5). Details of the hydrophobic pocket and the hydrophobic interfaces of the CaM C-lobe in CaM-CaMBD2-b and CaM-CaMBD2-a.

(A & B) Different amino acid residues contribute to formation of the hydrophobic pocket of the CaM C-lobe in CaM-CaMBD2-b (A) and CaM-CaMBD2-a (B). L428 is the anchor residue from CaMBDs.

(C & D) The C-lobe-CaMBD complexes are aligned at the N-terminal region of the CaMBD2-b and CaMBD2-a peptides. Constrains imposed by CaMBD2-b forces the CaM C-lobe to rotate approximately 180° and has led to formation of the C-lobe hydrophobic interface by different helices. W432, for instance, interacts with helix V in CaM-CaMBD2-b (C) instead of helix VII in CaM-CaMBD2-a (D).

Figure S5 (related to Figure 6). Canonical EF-hands.

(A) Sequences of the loop region of four EF-hands of CaM and their Ca^{2+}-coordinating amino acid residues (red).

(B) Structure of CaM EF-hand 2 in its Ca^{2+}-bound form.

Figure S6 (related to Figure 7). AEDANS labeled CaM in binding assays

(A) A simplified binding scheme depicting two separate steps in formation of the 2x2 CaM-CaMBD complex in the presence of Ca^{2+}, binding of Ca^{2+} to CaM, governed by the rate constant K_1, and binding of CaMBD to Ca^{2+}-bound CaM, governed by the rate constant K_2. Reduction in K1 or/and K2 will result in a right-ward shift of the dose-response curve for Ca^{2+}-dependent formation of the CaM-CaMBD2-b complex (e.g. Figure 7A).

(B) Residue T34 CaM is mutated to a cysteine for ADEANS labeling. T34 does not directly interfere with binding of Ca^{2+} or interaction with CaMBDs.

(C & D) Emission spectra (400-600 nm) of AEDANS labeled CaM (T34C) in complex with CaMBD2-b (B) or CaMBD2-a (C) at the indicated free Ca^{2+} concentrations.

Supplemental Table

Table S1 (related to Figure 3). Thermodynamic parameters for CaM-CaMBDs interactions in the presence of Ca^{2+} measured by ITC

CaM-CaMBD2-b	
K (M^{-1})	$1.26 \times 10^8 \pm 3.38 \times 10^7$
N	0.86 ± 0.07
ΔH (kcal mol^{-1})	-10.17 ± 2.64
ΔS (cal mol^{-1} K^{-1})	2.33 ± 0.92

CaM-CaMBD2-a	
K_1 (M^{-1})	$1.90 \times 10^8 \pm 2.83 \times 10^7$
N_1	0.40 ± 0.01
ΔH_1 (kcal mol^{-1})	-34.73 ± 0.96
ΔS_1 (cal mol^{-1} K^{-1})	-80.5 ± 3.39
K_2 (M^{-1})	$9.95 \times 10^6 \pm 1.76 \times 10^6$
N_2	0.45 ± 0.01
ΔH_2 (kcal mol^{-1})	16.18 ± 0.69
ΔS_2 (cal mol^{-1} K^{-1})	87.2 ± 1.98

Both CaM and CaMBDs were dialyzed into the same Ca^{2+} buffer prior to the experiments. Ca^{2+}-bound Ca^{2+} was titrated into the peptide solution. For CaM-CaMBD2-a, K_1 represents the rising phase, which is ethalpically favored, whereas K_2 corresponds to the declining phase, which is entropy-driven.

Table S2. Free-energy changes (in kcal/mol) via TI for Ca^{2+} annihilation.

	N-lobe			C-lobe			
Frame #	ΔG_{vdw}	ΔG_{elec}	ΔG	ΔG_{vdw}	ΔG_{elec}	ΔG	$\Delta\Delta G$
2000	-42.95	474.48	431.53	-42.78	459.01	416.23	15.30
4000	-42.98	484.63	441.65	-41.82	450.59	408.77	32.88
6000	-43.64	464.66	421.02	-41.51	459.81	418.30	2.72
8000	-43.84	480.66	436.82	-41.70	455.85	414.15	22.67
Mean	-43.35	476.11	432.76	-41.95	456.32	414.36	18.39
sem	0.23	4.35	2.09	0.28	2.09	2.05	6.35

vdW = van der Waals; elec = electrostatic

Supplemental Experimental Procedures

Crystallization and Structure Determination

The crystals of the CaM-CaMBD2-b complex were grown in hanging drops by vapor diffusion at 4 °C. The complex (1mM) was mixed in a 2:1:1 ratio with reservoir solution, which consists of (in M) Li_2SO_4 0.75, $(NH_4)_2SO_4$ 0.5, sodium citrate 0.1 (pH 4.65), and Silver Bullets reagent 70 at pH 6.8 (McPherson and Cudney, 2006). Crystals grew as thin plates within 3 weeks. Crystals were flash-cooled in liquid nitrogen for data collection, after a brief transfer to a cryoprotectant (1 part reservoir solution, 1 part Silver Bullets reagent 70, and 1 part 100% glycerol).

X-ray diffraction data were collected from a single crystal at Beamline X-29A of the National Synchrotron Light Source (Brookhaven National Laboratory) and processed using XDS (Kabsch). Initial phases were determined by molecular replacement (MR) using PHASER from the CCP4 suite (Collaborative Computational Project, 1994). The MR search model was the N-lobe of CaM and a portion of CaMBD2-a derived from the CaM-CAMBD2-a complex structure (1G4Y) (Schumacher et al., 2001). MR phases were improved using wARP (Perrakis et al., 1997), and automated model building was then performed in BUCANEER (Collaborative Computational Project, 1994), provided with a truncated version of the molecular replacement solution and improved MR phases from wARP. The crystallographic model was further constructed through iterative rounds of manual model building using Coot and crystallographic refinement using REFMAC5 and PHENIX (Adams et al., 2010; Collaborative Computational Project, 1994).

Eight calcium atoms were modeled in the region of the EF-hands based on strong positive peaks in difference Fourier electron density maps and the appropriate ligand geometry provided by the surrounding amino acid residues (Figure S1C). Anomalous difference Fourier

peaks were observed at these sites in electron density maps calculated with x-ray data collected at 1.74 Å, consistent with calcium binding at these sites. A phenylurea (PHU) molecule, one of the compounds in Silver Bullets reagent 70, was modeled in a hydrophobic pocket of the N-lobe of CaM based on strong electron density in difference Fourier maps and successful refinement of the coordinates. Due to the variety of small molecules contained within the crystallization condition, it was not possible to unambiguously model all solvent molecules observed in the electron density maps.

Several lines of evidence suggest that the presence of PHU in the crystallization solution, at the final concentration of 0.1%, is not the reason for the dramatic structural differences between CaM-CaMBD2-b and CaM-CaMBD2-a. First, the CaM-CaMBD2-b complex behaves differently from the CaM-CaMBD2-a complex in solutions without PHU, e.g. Figures 3 & 7. Second, binding assays, using fluorophore-labeled CaM and CaMBDs, show that PHU (0.1%) does not alter the apparent affinity of CaMBD2-b for CaM or the Ca^{2+}-dependent interactions between CaMBD2-a and CaM. Finally, the diffraction data of the protein crystals of CaM-CaMBD2-a obtained in the presence of PHU show that PHU is identified at the same location in the CaM N-lobe of the CaM-CaMBD2-a complex and that PHU does not cause any significant changes in the CaM-CaMBD2-a structure compared to 1G4Y.

Crystallographic statistics for data collection and model refinement are summarized in Table 1. Graphics of our structure, as well as that of 1G4Y and 1K90, were created using PyMol (Schrödinger, LLC). Structural comparisons were made using Coot, CCP4 or PyMol. Additional structural analyses, such as the buried surface area (BSA), were performed, online, at

http://www.ebi.ac.uk/pdbsum.

Electrophysiology

Both KCNN2-a and KCNN2-b, along with CaM, were subcloned into the pCDNA3.1 expression vector (Invitrogen) and expressed in TsA201 cells cultured in the Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum plus penicillin and streptomycin. A calcium phosphate method was used for transfection of KCNN2-a or KCNN2-b, together with CaM and GFP at a ratio of 5:2.5:1 (weight). Channel activities were recorded 1 – 2 days after transfection.

Patch clamp experiments were performed with a Multiclamp 700B amplifier (Molecular Devices) at room temperature. pClamp 10.2 (Molecular Devices) was used for data acquisition and analysis. The resistance of the patch electrodes ranged from 3–7 MΩ. The pipette solution contained (in mM): KCl 140, HEPES 10, $MgSO_4$ 1, at pH 7.4. The bath solution contained (in mM): KCl 140, and HEPES 10, at pH 7.2. EGTA (1 mM), HEDTA (1 mM) and Dibromo-BAPTA (0.1 mM) were mixed with Ca^{2+} to obtain the final free Ca^{2+} concentrations in various bath solutions using the software by Chris Patton of Stanford University. The Ca^{2+} concentrations were verified using Fluo-4 and standard Ca^{2+} buffers (Molecular Probes).

Currents were recorded using an inside-out patch configuration. The intracellular face was initially exposed to a zero-Ca^{2+} bath solution, and subsequently to bath solutions with increasing Ca^{2+} concentrations to activate SK2 channels. Currents were recorded by repetitive 1 sec-voltage ramps from -100 mV to +100 mV from a holding potential of 0mV. One minute after switch bath solutions, ten sweeps, with a one-second interval, were recorded at each Ca^{2+}

concentration. The integrity of the patch was examined by switching the bath solution back to the zero-Ca^{2+} buffer. Data from patches, which did not show significant changes in seal resistance after solution changes, were used for further analysis. To construct the Ca^{2+} dependent channel activation curves, the current amplitude at -100mV in response to various Ca^{2+} concentrations was normalized to that obtained at 10 μM Ca^{2+} for both KCNN2-a and KCNN2-b. Relative currents at -100 mV were plotted as a function of Ca^{2+} concentrations. Half-activation Ca^{2+} concentration (EC50) and Hill coefficients were determined by fitting the data points obtained from individual experiments to a standard dose-response curve (y = 100/(1 + (x/EC50)^-Hill)).

Thermodynamic integration (TI) in MD simulation.

Each TI sampling MD simulation was 40,000 steps with a time-step of 2 fs, and ensemble averaging was performed from the final 37,500 time-steps. The discrete alchemical pathway connecting the Ca^{2+}-bound state to a Ca^{2+}-free state (i.e., Ca^{2+} annihilation) comprised 19 images with order parameter values reported in Table S2. In all MD simulations, bond lengths were held rigid using the RATTLE algorithm. Results from all eight TI calculations (Ca^{2+} unbinding from the N- and C-lobes from four independent configurations) are shown in Table S2.

Table S3. Schedule of values for TI order parameters λ_{vdW} and λ_{elec}.

Alchemical image	λ_{vdW}	λ_{elec}
1 (Ca^{2+}-bound)	1.0	1.0
2	0.99999	0.99999
3	0.9999	0.9999
4	0.999	0.999
5	0.99	0.99
6	0.9	0.89
7	0.8	0.78

8	0.7	0.67
9	0.6	0.56
10	0.5	0.44
11	0.4	0.33
12	0.3	0.22
13	0.2	0.11
14	0.1	0.0
15	0.01	0.0
16	0.001	0.0
17	0.0001	0.0
18	0.00001	0.0
19 (Ca^{2+}-free)	0.0	0.0

vdW = van der Waals
elec = electrostatic

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