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Backbone and Side-Chain Resonance Assignments of $(\text{Ca}^{2+})_4$ -Calmodulin Bound to Beta Calcineurin A CaMBD Peptide

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Abstract

Calcineurin (CaN) is a heterodimeric and highly conserved serine/threonine phosphatase (PP2B) that plays a critical role in coupling calcium signals to physiological processes including embryonic cardiac development, NF-AT-regulated gene expression in immune responses, and apoptosis. The catalytic subunit (CaN_A) has three isoforms (α , β , and γ ,) in humans and seven isoforms in *Paramecium*. In all eukaryotes, the EF-hand protein calmodulin (CaM) regulates CaN activity in a calcium-dependent manner. The N- and C-domains of CaM (CaM_N and CaM_C) recognize a CaM-binding domain (CaMBD) within an intrinsically disordered region of CaN_A that precedes the auto-inhibitory domain (AID) of CaN_A. Here we present nearly complete ¹H, ¹³C, and ¹⁵N resonance assignments of $(\text{Ca}^{2+})_4$ -CaM bound to a peptide containing the CaMBD sequence in the beta isoform of CaN_A ($\beta\text{CaN}_A\text{-CaMBDp}$). Its secondary structure elements predicted from the assigned chemical shifts were in good agreement with those observed in the high-resolution structures of $(\text{Ca}^{2+})_4$ -CaM bound to CaMBDs of multiple enzymes. Based on the reported literature, the CaMBD of the α isoform of CaN_A can bind to CaM in two opposing orientations which may influence the regulatory function of CaM. Because a high resolution structure of $(\text{Ca}^{2+})_4$ -CaM bound to $\beta\text{CaN}_A\text{-CaMBDp}$ has not been reported, our studies serve as a starting point for determining the solution structure of this complex. This will demonstrate the preferred orientation of $(\text{Ca}^{2+})_4$ -CaM on the CaMBD as well as the orientations of CaM_N and CaM_C relative to each other and to the AID of βCaN_A .

Keywords

Molecular recognition; allostery; EF-hand protein; calcium-mediated signal transduction; phosphatase

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Biological context

Calcineurin (CaN), a heterodimeric Ser/Thr phosphatase (Klee and Krinks 1978, Stewart, Ingebritsen et al. 1982, Rusnak and Mertz 2000), plays an essential role in diverse cellular responses to influxes of calcium. By dephosphorylating intracellular substrates, CaN regulates numerous signaling pathways that contribute to both pathological and normal developmental processes in eukaryotes. These include exocytosis and backward swimming in *Paramecia* (Fraga, Sehring et al. 2010), regulation of environmental response to stress in fungi (Cyert 2003, Kraus and Heitman 2003), cardiac development (Petrashkevskaya, Bodi et al. 2002), hypertrophy (Molkentin, Lu et al. 1998, Sussman, Lim et al. 1998), neuronal plasticity (Wang and Kelly 1997, Foster, Sharrow et al. 2001), and T-cell activation in mammals. The activity of CaN is the target of immuno-suppressive drugs including cyclosporin and tacrolimus (Liu, Farmer et al. 1991).

Although CaN activity is required for multiple pathways during development, embryonic heart development is the earliest and most sensitive responder to changes in CaN activity. To regulate heart development, CaN dephosphorylates cytoplasmic transcription factors in the NF-AT family (Hogan, Chen et al. 2003) that enter the nucleus and launch a transcription program that controls cardiac muscle growth by regulating *Nkx2-5* (Allen, Allen-Brady et al. 2006). However, how this process is regulated by fluxes in intracellular calcium levels is poorly understood.

In all eukaryotes, CaN is composed of a large (61 kDa) catalytic subunit CaN_A and a smaller (19 kDa) constitutively bound regulatory subunit CaN_B. CaN is unusual in requiring two distinct EF-hand Ca²⁺-binding proteins for regulation. Its intrinsic subunit CaN_B contains 4 EF-hand (helix-loop-helix) Ca²⁺-binding sites and is bound constitutively to CaN_A. An additional modulator that is recruited to bind to CaN in response to rising calcium levels is calmodulin (CaM, 17 kDa), which also contains 4 EF-hand Ca²⁺-binding sites, and is an essential eukaryotic calcium sensor. Both CaN_B and CaM increase the activity of CaN_A. CaN_B primarily decreases the K_m with a smaller effect on V_{max} , while CaM primarily increases V_{max} (Perrino, Ng et al. 1995). There are three vertebrate isoforms of CaN_A (α , β , and γ), and two isoforms of CaN_B (1 and 2). Proteolytic and structural studies show that the C-terminal region of CaN_A has multiple regulatory regions including a CaN_B-binding site, a calmodulin-binding domain (CaMBD), an “intrinsically disordered” (ID) region, and a short, helical region called the auto-inhibitory domain (AID) that occludes the active site (Hubbard and Klee 1989, Hashimoto, Perrino et al. 1990).

Under resting conditions of low calcium (10 to 100 nM), CaN_A is inhibited by the AID. Activation of CaN requires release of the AID which is mediated by Ca²⁺ binding to CaN_B and CaM. However, the molecular mechanism of CaN activation is not fully understood because the primary binding site for CaM is within an intrinsically disordered region on CaN_A that has not been observed in high resolution structures of the catalytic domain of CaN_A. There are crystallographic structures of (Ca²⁺)₄-CaM bound to a peptide representing the CaMBD sequence of the α isoform of CaN_A (Ye, Wang et al. 2008, Dunlap, Guo et al. 2014), but no structures were reported for (Ca²⁺)₄-CaM bound to the CaMBD of the β isoform of the CaN_A (β -CaN_A).

Here we report the NMR assignments of ^{13}C , ^{15}N -(Ca^{2+})₄-CaM bound to a ^{13}C , ^{15}N -labeled peptide corresponding to the CaMBD of βCaN_A . This peptide is referred to hereafter as βCaN_A -CaMBDp. These assignments lay the foundation for determining the solution structure of this complex for further understanding thermodynamic and enzymatic studies that probe the CaM- CaN_A interface using mutational perturbations of both CaM and CaN_A , and for better understanding of the role of each calmodulin domain in the mechanism of calcium-triggered activation of CaN.

Methods and experiments

Protein expression and purification

Complexes of calcium-saturated CaM bound to βCaN_A -CaMBDp were obtained through bacterial co-expression of CaM and a GST-tagged form of the βCaN_A CaMBD sequence. A pT7-7 vector (ampicillin^R) expressed full-length (148 a.a.) wild-type CaM (*Paramecium* sequence, 89% identical to mammalian CaM) (Jaren, Kranz et al. 2002). A pBG101 vector (kanamycin^R) containing an N-terminal GST-tag and 3C protease cleavage site (Damo, Feldkamp et al. 2013) was a generous gift from Walter J. Chazin, Director of the Center for Structural Biology at Vanderbilt University. The vector was modified commercially (Mutagenex) to insert the βCaN_A CaMBD (residues 397–426, [SAAARKEIIRNKIRAIGKMARVFSVLREES]) following the 3C protease cleavage site.

The plasmids were co-transformed into *E. coli* BL21 (DE3) cells. Cultures were grown in LB media at 37 °C to an OD₆₀₀ of 0.7, pelleted and exchanged into minimal media containing $^{15}\text{NH}_4\text{Cl}$ (CIL) and either unlabeled glucose or $^{13}\text{C}_6$ -glucose (CIL). After an hour of incubation at 37 °C, cells were induced with 0.8 mM IPTG (Marley, Lu et al. 2001) and grown for 4 more hours. The harvested cells were then lysed by sonication and pelleted at 20,000 rpm for 50 min at 4 °C. Supernatant containing GST-fusion protein with bound CaM was rocked with Glutathione Sepharose resin (Amersham Biosciences) at 4 °C. Resin was then transferred to a column (10 mL), washed with 5 CV (column volumes) of buffer A (50 mM HEPES, 100 mM KCl, 1 mM MgCl_2 , 50 μM EGTA, 10 mM CaCl_2 , 0.01% (w/v) NaN_3 , pH 7.4) at 4 °C, followed by 5 CV of a matching buffer with higher salt (500 mM KCl) at 4 °C. The resin was treated with rhinovirus 3C protease (Damo, Feldkamp et al. 2013) in buffer A, and incubated for 3 h at 22 °C with gentle nutation. The released, soluble complex of (Ca^{2+})₄-CaM bound to βCaN_A -CaMBDp was collected, concentrated and exchanged into an NMR buffer containing 10 mM imidazole, 100 mM KCl, 0.01% (w/v) NaN_3 , 5 mM CaCl_2 , pH 6.5 (4 °C). After 3C protease cleavage, the peptide contains a 4-residue N-terminal tag (G-P-G-S) at the N-terminus of the βCaN_A CaMBD sequence. They were annotated as −4 to −1 in the BMRB deposit. The complete peptide sequence used in our experiments was GPGSSAAARKEIIRNKIRAIGKMARVFSVLREES. Samples used in the NMR experiments were >95% pure as determined by denaturing electrophoresis, UV/Vis spectroscopy, and reversed phase HPLC. Protein concentrations were determined using the BCA assay (Pierce Biotechnology; Rockford IL).

NMR Spectroscopy

All NMR experiments were acquired at 298 K on a 4-channel 600 MHz Varian Inova NMR spectrometer equipped with a triple resonance gradient probe. Samples used in the NMR experiments, either uniformly ^{13}C , ^{15}N -labeled or unlabeled, contained 780 to 900 μM CaM: βCaN_A -CaMBDp complex (1:1 ratio) in 10 mM d_4 -imidazole, 5 mM CaCl_2 , 100 mM KCl, 0.01% NaN_3 at pH 6.5. All spectra were processed with NMRPipe (Delaglio, Grzesiek et al. 1995) and analyzed with CCPN Analysis (Vranken, Boucher et al. 2005).

$(\text{Ca}^{2+})_4$ -CaM binds the CaMBD of βCaN_A tightly, with a K_d of ~ 1 pM (O'Donnell, Yu et al. 2011). Thus, the complex is in slow exchange on the NMR timescale. A uniformly ^{13}C , ^{15}N -labeled sample was used to assign the backbone and aliphatic sidechains using the following experiments: $^1\text{H}/^{15}\text{N}$ HSQC, HNCACB, CBCA(CO)NH, HNCO, HBHA(CO)NH, and C(CO)NH for the sample in 90% $\text{H}_2\text{O}/10\%$ D_2O as well as $^1\text{H}/^{13}\text{C}$ HMQC, HCCH-TOCSY, and HCACO for the sample in 100% D_2O . Aromatic sidechain chemical shifts were assigned using the following experiments: (HB)CB(CGCD)HD, (HB)CB(CGCDCE)HE, and aromatic $^1\text{H}/^{13}\text{C}$ CT-HSQC on a uniformly ^{13}C , ^{15}N -labeled sample plus 2D gCOSY, 2D TOCSY, and 2D NOESY on an unlabeled sample in 100% D_2O . Assignments were further confirmed and extended using 3D ^{13}C -edited NOESY (for ^{13}C , ^{15}N -labeled sample in 100% D_2O) and 3D ^{15}N -edited NOESY (for ^{15}N -labeled sample in 90% $\text{H}_2\text{O}/10\%$ D_2O).

Secondary structure and order parameters of $(\text{Ca}^{2+})_4$ -CaM and βCaN_A -CaMBDp were predicted from the assigned chemical shifts using TALOS+ (Shen, Delaglio et al. 2009). Chemical shift perturbations of CaM upon βCaN_A -CaMBDp binding were calculated against the chemical shifts of free $(\text{Ca}^{2+})_4$ -CaM (Jaren, Kranz et al. 2002), according to the following equation:

$$\Delta\delta = \sqrt{(\Delta\delta_{\text{HN}})^2 + (\Delta\delta_{\text{N}} \cdot 0.10134)^2}$$

where δ_{HN} and δ_{N} represent the observed chemical shift changes for $^1\text{H}_\text{N}$ and ^{15}N , respectively.

Extent of assignments and data deposition

The assigned ^1H - ^{15}N HSQC spectrum of the complex between CaM and βCaN_A -CaMBDp is shown in Figure 1. Clearly, the cross-peaks in the spectrum are reasonably well dispersed given the fact that the complex contains largely α -helical secondary structures. The backbone $^1\text{H}_\text{N}$ and ^{15}N assignments of the non-proline residues in the complex are complete except for the two residues at the N-terminus of CaM (residues A1, E2) and one residue at the N-terminus of the peptide (residue G(-4)). The sidechain NH_2 of Asn and Gln, as well as Arg NH_ϵ , are completely assigned in both molecules of the complex.

Aliphatic sidechain assignments for CaM are >99% complete for ^{13}C resonances and at least 95% complete for ^1H resonances; 27 of the 31 missing aliphatic protons are methylenes which are likely degenerate. The aromatic sidechain ^1H and ^{13}C assignments are nearly 100% complete for CaM and the only unassigned aromatic carbon is F16 C ζ .

The β CaN_A-CaMBDp aliphatic sidechain assignments are 94% complete for ^{13}C resonances and at least 86.5% complete for ^1H resonances. Residue K408 gives rise to particularly poor signals in a number of experiments and accounts for many of the missing peptide assignments: 3 of 7 total unassigned ^{13}C resonances and 7 of 22 unassigned ^1H resonances are in this residue. Of the remaining 15 unassigned aliphatic protons, 14 are likely degenerate methylenes. F419, the sole aromatic residue in the peptide, is completely assigned.

The predicted order parameters (Figure 2A, 2B) and secondary structure elements (Figure 2C, 2D) for CaM and β CaN_A-CaMBDp were calculated with TALOS+ (Shen, Delaglio et al. 2009) using $^1\text{H}_\text{N}$, ^{15}N , $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$, $^{13}\text{C}'$, and $^1\text{H}_\alpha$ chemical shifts. The predicted pattern of secondary structure for CaM is consistent with known structures of calcium-saturated calmodulin such as 1EXR.pdb (Wilson and Brunger 2000). As illustrated in Figure 2A, the CaM_N (blue) and CaM_C (red) each have 4 α -helices, resulting in a total of 8 α -helices, while a linker (gray) between the domains is disordered, and a short β -sheet (consisting of β 1 and β 2 in CaM_N, or β 3 and β 4 in CaM_C) connects the two paired EF-hands (helix-loop-helix motifs) within each domain. Residues with reduced order parameters map largely to the termini and interhelical segments of CaM. β CaN_A-CaMBDp is predicted to be a single long α -helix with disordered ends, which is consistent with known calmodulin binding domains that form basic, amphipathic α -helices.

Backbone amide chemical shift perturbations (CSPs) for $(\text{Ca}^{2+})_4$ -CaM upon binding to β CaN_A-CaMBDp when compared to free $(\text{Ca}^{2+})_4$ -CaM (Jaren, Kranz et al. 2002) were calculated and depicted in Figure 2E. The horizontal line corresponds to the average change (0.203 ppm) in chemical shift (SD of 0.196). The largest CSPs were concentrated in the linker connecting CaM_N and CaM_C, suggesting that the domains reorient relative to one another upon peptide binding. CSPs spread throughout the sequence of $(\text{Ca}^{2+})_4$ -CaM, indicating that β CaN_A-CaMBDp interacts with both domains. However, the CSPs are larger and more numerous in CaM_N.

CaMBD peptides may bind to CaM in either one of two orientations (i.e., parallel or anti-parallel), and the peptide-binding orientation may influence the functional roles of the domains of CaM in activating CaN. In our earlier study, we provided a structural model of CaM in complex with β CaN_A-CaMBDp based on preliminary intermolecular NOE analyses of ^{13}C , ^{15}N - $(\text{Ca}^{2+})_4$ -CaM when bound to an unlabeled peptide (O'Donnell, Yu et al. 2011). With the assignments reported here, we plan to determine the solution structure of this complex, and to reveal in detail the orientations of CaM_N and CaM_C when both are bound to β CaN_A-CaMBDp and their orientation relative to the AID of β CaN_A.

The backbone and sidechain NMR assignments were deposited with the BioMagResBank (BMRB <http://www.bmr.bwisc.edu>, accession number 27017).

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Figure 1.

^1H - ^{15}N HSQC of $(\text{Ca}^{2+})_4\text{-CaM}$ in complex with $\beta\text{CaN}_\text{A}\text{-CaMBDp}$. Peak assignments are shown on the spectrum. The area in panel A surrounded by the dashed box is expanded for clarity in panel B. Gray contours indicate aliased arginine sidechain crosspeaks. Resonance labels for CaM are in black and those for the peptide are in green. Dashed lines connect Asn and Gln sidechain NH_2 crosspeaks. The dashed arrow indicates that the upfield $\text{H}\delta$ of N407 is outside the displayed spectral region. For the peptide, negative numbers denote non-native residues preceding the sequence corresponding to the CaMBD of βCaN_A .

Figure 2.

Predicted order parameters (**A,B**), and normalized chemical shift index (CSI) indicative of secondary structure types (**C,D**) calculated with TALOS+ from the assigned chemical shifts for both $(Ca^{2+})_4$ -CaM (**A,C**) and β CaNA-CaMBDp (**B,D**) in the complex. The inset in panel **A** depicts regions of CaM: 4 calcium-binding loops (yellow), residues in the N-lobe (blue), residues in the C-lobe (red), and linkers between the EF-hands (gray); the inset bar (green) in panel **B** corresponds to residues in β CaNA-CaMBDp. The histogram (**E**) depicts chemical shift perturbations ($\delta\delta$, ppm) for CaM upon binding β CaNA-CaMBDp, calculated as described in the methods section. The horizontal line corresponds to the average change (0.203 ppm) in chemical shift perturbation.