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Abstract

Calcineurin (CaN) plays an important role in T-cell activation, cardiac system development and nervous system function. Previous studies have demonstrated that the regulatory domain (RD) of CaN binds calmodulin (CaM) towards the N-terminal end. Calcium-loaded CaM activates the serine/threonine phosphatase activity of CaN by binding to the RD, although the mechanistic details of this interaction remain unclear. It is thought that CaM binding at the RD displaces the auto-inhibitory domain (AID) from the active site of CaN, activating phosphatase activity. In the absence of calcium-loaded CaM, the RD is disordered, and binding of CaM induces folding in the RD. In order to provide mechanistic detail about the Ca…aN interaction, we have undertaken an NMR study of the RD of CaN. Complete ^{13}C, ^{15}N and ^{1}H assignments of the RD of CaN were obtained using solution NMR spectroscopy. The backbone of RD has been assigned using a combination of ^{13}C-detected CON-IPAP experiments as well as traditional HNCO, HNCA, HNCOCA and HNCACB-based 3D NMR spectroscopy. A ^{15}N-resolved TOCSY experiment has been used to assign Hα and Hβ chemical shifts.

Keywords

Calcineurin; Intrinsically Disordered Protein; Calmodulin Binding

Biological Context

Calcineurin (CaN), also known as protein phosphatase 2B, is a Ca^{2+} and calmodulin (CaM)-dependent serine/threonine phosphatase protein (Wang and Desai 1976). CaN participates in various roles associated with cardiac, vascular, and nervous system development, making CaN-mediated signaling very important for learning and memory, skeletal muscle growth,

and immune system activation (Rusnak and Mertz 2000). Because of its roles in these signaling pathways, dysregulation of CaN has been implicated in many pathological states, e.g. Alzheimer's disease (Ermak and Davies 2013), Down syndrome (Hoeffer, Dey et al. 2007), and cardiac hypertrophy (Vega, Bassel-Duby et al. 2003). CaN has the ability to dephosphorylate and activate the nuclear factor of activated T-cells (NFAT). NFAT proteins are a family of transcription factors that activate genes involved in immune response (Macian 2005). CaN is therefore an important target for immunosuppressant drugs like cyclosporin A (CspA) and FK506 (Liu, Farmer et al. 1991).

CaN is a heterodimer consisting of a 60-kDa A chain and a 19-kDa B chain. There are three isoforms of the CaN A chain: the α-isoform (the dominant form in neurons), the β-isoform (broadly distributed) and the testis-specific γ-isoform (Rusnak and Mertz 2000). The CaN A chain consists of the catalytic domain, the B-chain binding domain, the regulatory domain (RD, residues 388–458), the autoinhibitory domain (AID), and a short C-terminal tail (Hubbard and Klee 1989). The B chain is homologous to CaM and binds four calcium ions (Stemmer and Klee 1994). The AID keeps CaN in an inactive state at low concentrations of Ca^{2+}, but at increased levels, CaM binds calcium and then binds to the calmodulin-binding region (CaMBR) of CaN, located in the RD. In the absence of calcium-loaded CaM, the RD is disordered (Manalan and Klee 1983, Yang and Klee 2000, Rumi-Masante, Rusinga et al. 2012). Binding of CaM to CaN causes a structural ordering in the RD that removes the AID from the catalytic site of CaN, thereby activating the enzyme (Shen, Li et al. 2008, Dunlap, Cook et al. 2013, Dunlap, Guo et al. 2014, Zhao, Yang et al. 2014).

Several three-dimensional structures of CaN have been obtained at high resolution by X-ray diffraction methods as a means of understanding its catalytic mechanism. The original structure included residues 21–372 of CaN complexed with the inhibitory compound FK506 (Griffith, Kim et al. 1995). Subsequent work has investigated CaN bound to cyclosporin A (Jin and Harrison 2002), and a solution NMR structure has been obtained for the catalytic domain of CaN bound to an NFAT-derived PVIVIT peptide (Takeuchi, Sun et al. 2015). The structure of the CaM-CaN complex is of high interest (Ye, Wang et al. 2008, Ye, Feng et al. 2013, Dunlap, Guo et al. 2014); however, no structure to date has been obtained that includes the complete CaN RD. Here, we present backbone and side-chain assignments of the RD of CaN (residues 388–468) in the solution phase, obtained using 2D and 3D NMR techniques. The chemical shifts support a largely disordered conformation, although secondary shifts suggest that some helical propensity may be present in the RD in the absence of Ca^{2+}-loaded CaM.

Methods and Experiments

RD Expression and Purification

The calmodulin-containing pETCaMI plasmid, and the RD-containing pETRD plasmid were co-transformed into *Escherichia coli* BL21(Star) DE3 competent cells (Invitrogen) for expression. In this system, the Human Calcineurin A domain (Isoform 1) RD protein sequence (residues 388–468) is flanked by three residues on the N-terminus (MAG) and a C-terminal Histidine tag (GWGGGLEHHH HHH). For simplicity, the first three residues (385–387) and the C-terminal Histidine tag residues (469–481) are numbered as part of the

sequence, even though they are not part of the wild-type RD domain. The total RD construct, containing 97 residues, corresponds to constructs used previously for the study of the RD, and purification proceeded as described previously (Dunlap, Cook et al. 2013, Dunlap, Guo et al. 2014). Transformed cells were incubated in 100 ml terrific broth (TB; 12 g/L tryptone, 24 g/L yeast extract, 4.0 ml glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4, 75 μg/ml ampicillin and 50 μg/ml kanamycin) overnight at 37°C. These starter cultures were then added to 1L of M9 minimal media (containing 75 μg/ml ampicillin and 50 μg/ml kanamycin). The starter culture in TB was used to inoculate the M9 media such that the initial OD_{600} was 0.05. This larger culture was incubated at 37°C in a 200 rpm shaker. When the culture reached an OD_{600} of 0.6–0.7, expression was induced with a final concentration of 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and harvested after 4 hours (OD_{600} ≈ 1.6). These cells were pelleted by centrifugation for 30 minutes at $8,000 \times g$, and then re-suspended in lysis buffer (20mM HEPES pH 7.5, 200 mM NaCl, 0.1mM PMSF, 125μg/ml lysozyme). One tablet of Roche Complete EDTA-free protease inhibitor was added to the lysis buffer, and the re-suspended cells were sonicated on ice in a Branson Sonifier 250 at power level 6 for 3 repetitions of a 2 minute pulse/2 minute rest. Processed lysate was centrifuged at $18,000 \times g$ for 45 minutes at 4°C, with CaN remaining in the soluble fraction. The CaN RD was purified on a Ni-NTA column (GE Life Sciences) using urea-thiourea buffer to separate the co-expressed CaM protein from the RD. The Ni-NTA column was equilibrated with 5 column volumes (CV) of 7M urea/thiourea buffer (10mM HEPES pH 7.5, 200mM NaCl, 2M thiourea, 5M urea) at room temperature. Centrifuged lysate mixed with 3X volume of urea/thiourea buffer for 10 minutes and then applied to the Ni-NTA column. The urea/thiourea concentration was reduced to zero by washing the column with binding buffer (20mM HEPES pH 7.5, 200mM NaCl). The protein was eluted with 20mM HEPES pH 7.5, 200mM NaCl, 2mM $CaCl_2$ and 250mM imidazole. Eluted protein was mixed with 50ml of column buffer (20mM HEPES pH 7.5, 200mM NaCl, 2mM $CaCl_2$). Final purification was performed with a CaM-Sepharose column (GE Life Sciences) equilibrated with 5 CV of column buffer. Protein was eluted with a gradient from 0 – 100% elution buffer (20mM Tris pH 7.5, 200mM NaCl, 2mM EGTA) and pooled protein fractions (where the absorbance at 280 nm was higher than at 260 nm) were dialyzed in dialysis buffer (20 mM PIPES pH 5.5, 10 mM DTT and 100 mM NaCl) overnight and stored at 4°C. The molecular weight and purity of the 97-residue CaN RD were confirmed by LC-MS and SDS-PAGE.

NMR Spectroscopy

All samples for NMR experiments were prepared in 20mM PIPES (pH 5.5), 100 mM NaCl, 6% D_2O and 10 mM DTT. Protein concentrations ranged from 0.2 to 0.25 mM for all NMR experiments in a total volume of 550 μL. The samples were transferred into 5 mm NMR sample tubes and stored at 4°C until required for NMR experiments. All the NMR experiments were performed on a Bruker AVANCE III 600 MHz spectrometer, equipped with a multinuclear biomolecular (QCI) cryo-probe. All NMR spectra were acquired at 288 K using Bruker TopSpin software. Experimental spectra were processed with NMRPipe (Delaglio, Grzesiek et al. 1995). Processed NMR spectra were assigned and visualized using Sparky.

Protein backbone assignments were made using 3D HNCO, HNCA, HNCOCA CACBCONH, CBCANH and [^{1}H-^{15}N]-HSQC experiments (Grzesiek and Bax 1992). Side chain ^{1}H and ^{13}C assignments were obtained using a 3D TOCSY-HSQC and 3D HCCCONH spectra (Schleucher, Schwendinger et al. 1994, Carlomagno, Maurer et al. 1996). While the initial amide-proton resolved experiments were promising, spectral overlap and crowding precluded the complete assignment using ^{15}N-HSQC resolved experiments. In addition, the CaN RD contains three proline residues which are not detected in these spectra. Therefore, we also recorded an (HACA)N(CA)CON NMR experiment, which was instrumental in determining the complete backbone trace for this protein (Bastidas, Gibbs et al. 2015).

Assignments and Data Deposition

Complete backbone and side-chain ^{13}C and ^{15}N chemical shift assignments of RD CaN are presented here, and these assignments will provide insight into this disordered region of CaN. A 2D ^{1}H-^{15}N HSQC spectrum of the RD construct (residues 388–468, plus flanking residues), with assignments of the amide backbone, is shown (Fig. 1A). The chemical shifts of RD CaN have been deposited in the Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu) under the accession number 26990. The 2D and 3D NMR spectra enabled the detection of the backbone and aliphatic side-chain resonances for the CaN RD. In the absence of calcium loaded CaM, the RD is fully disordered, and chemical shifts exhibit sharp lines and narrow dispersion in the ^{1}H dimension (Fig. 1A), a characteristic typical of intrinsically disordered proteins (IDPs). Almost all non-proline residues in the construct have been assigned, except residues 385–387 (part of the N-terminal, non-RD flanking region), and residue R397 (likely because of spectral overlap). In addition, all three proline residues have been assigned in the CON-resolved spectra (Fig. 1B). Backbone sequential assignment was achieved for 95% of the amide resonances, 98% and 88% of the ^{13}C$_\alpha$ and ^{13}C$_\beta$ resonances respectively, and 97% of the ^{13}CO resonances. Sets of signals originating from the non-RD flanking regions were more challenging to assign, likely because of elevated hydrogen exchange near the N-terminus (Bai, Milne et al. 1993) and degeneracy in the Histidine tag near the C-terminus.

Deviations from random coil chemical shifts are indicative of transiently-formed structure in intrinsically disordered proteins. Sequence-dependent random coil shifts have been corrected for pH and temperature and suggest weak structural preferences in the RD (Kjaergaard, Brander et al. 2011). For example, ^{13}C$_\alpha$ chemical shifts (Fig. 2A) indicate a slight propensity for helix formation between residues 417–422 and 452–460, consistent with previous findings that indicate these regions adopt a helical conformation when bound to Calmodulin (Manalan and Klee 1983, Dunlap, Guo et al. 2014). This propensity, however, is not borne out by ^{13}C$_\beta$ chemical shifts (Fig. 2B), which are quite random throughout the sequence. Secondary structure propensity (SSP) scores (Marsh, Singh et al. 2006) and δ2D population predictions (Camilloni, De Simone et al. 2012) are also consistent with a highly disordered RD (Fig. 2C, 2D), although a weak helical score is observed for residues 417–422 and 452–460. The scores suggest less structural bias than has been observed in other IDPs, for example in the helical region of the ALS-related protein TDP-43 (Conicella, Zerze

et al. 2016). NMR assignments of the RD CaN will be valuable for future studies of CaN activation and its interaction with many target proteins.

Acknowledgments

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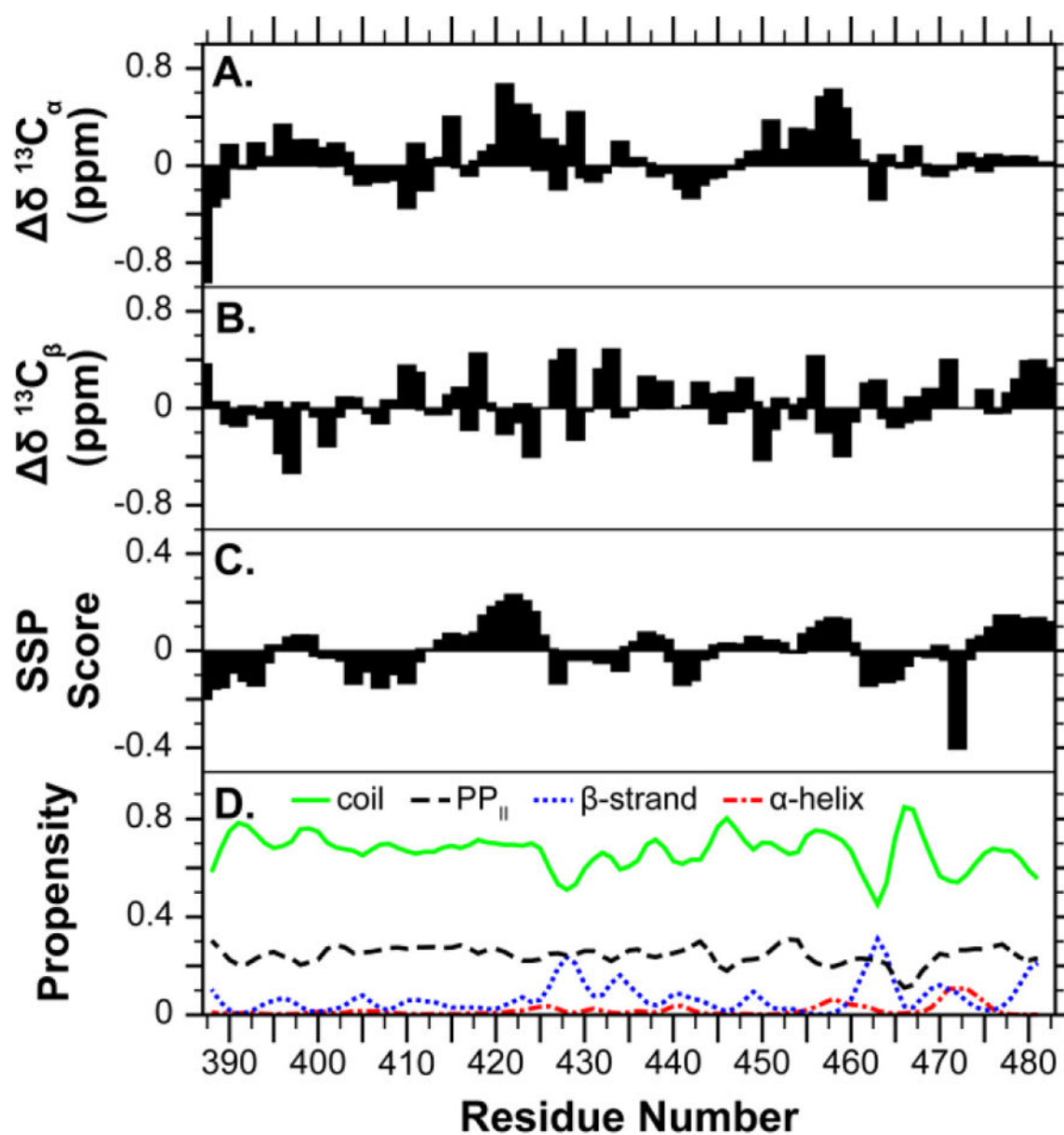
Figure 1.
Assigned [^{1}H-^{15}N]-HSQC spectrum (A) and (HACA)N(CA)CON spectrum (B) of CaN RD in solution. All three proline residues in panel B are aliased.

Figure 2.
$^{13}C\alpha$ (A) and $^{13}C\beta$ (B) secondary chemical shifts for the CaN RD. Secondary $^{13}C\alpha$ shifts suggest a slight propensity for helix formation between residues 417–422 and 452–460. SSP scores (C) for the RD are also weakly helical in these regions. δ2D-predicted secondary structure propensities (D) based on experimental RD chemical shifts confirm that the RD overall is highly disordered. Secondary structure classifications for δ2D are designated in the legend.