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¹H, ¹⁵N, and ¹³C Chemical Shift Assignments of Murine Calcium-binding Protein 4

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Abstract

Calcium-binding protein 4 (CaBP4) regulates voltage-gated Ca²⁺ channels in retinal rod cells and specific mutations within CaBP4 are associated with congenital stationary night blindness type 2. We report complete NMR chemical shift assignments of the Ca²⁺-saturated form of CaBP4 with Ca²⁺ bound at EF1, EF3 and EF4 (BMRB no. 18877).

Keywords

calcium; CaBP4; retina; EF-hand; CaV1.4

Biological Context

Neuronal calcium-binding proteins (CaBP1–8, (Haynes et al., 2012)) belong to a subclass of the calmodulin (CaM) superfamily (Ikura, 1996) and regulate particular Ca²⁺ channel targets in the brain and retina (Haeseleer et al., 2000). Multiple isoforms of CaBPs are localized in different neuronal cell types and perform specialized roles in signal transduction (Haynes et al., 2012; Haynes et al., 2004). The CaBP1 isoform regulates Ca²⁺-dependent activity of inositol 1,4,5-trisphosphate receptors (InsP3Rs) in the brain (Li et al., 2013; Yang et al., 2002), whereas CaBP4 regulates voltage-gated Ca²⁺ channels (CaV1.4) in retinal photoreceptor cells (Haeseleer et al., 2004). Mutations in both CaV1.4 (Mansergh et al., 2005) and CaBP4 (Aldahmesh et al., 2010) have been identified in patients suffering from congenital stationary night blindness type 2 (CSNB2), indicating that defects in Ca²⁺-dependent regulation of CaV1.4 by CaBP4 are closely associated with CSNB2. Indeed, deletion of CaBP4 in the mouse leads to a CSNB2-like phenotype (Haeseleer et al., 2004). Thus, understanding the structural interaction of CaBP4 with CaV1.4 may provide insights for developing therapeutic agents for treating CSNB2.

CaBP4 is structurally similar to CaBP1 and CaM (Fig. 1). Like CaBP1, CaBP4 contains two globular domains (N- and C-lobe) that each contains a pair of EF-hand motifs connected by a central linker (Haeseleer et al., 2000). The second EF-hand in CaBP4 lacks conserved residues in the binding loop and is predicted to not bind Ca²⁺ (Haeseleer et al., 2000). CaBP4 interacts structurally with the C-terminal region of CaV1.4 (Shaltiel et al., 2012) and this Ca²⁺-dependent interaction has been implicated in the modulation of voltage-dependent

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CaV1.4 activation (Haeseleer et al., 2004). We report here the NMR assignments of CaBP4 with Ca²⁺-bound at EF1, EF3 and EF4, as a first step toward elucidating its atomic-level structure and Ca²⁺-dependent regulatory mechanism of CaV1.4

Methods and Experiments

Expression and Purification of Mouse CaBP4

The full-length mouse CaBP4 (residues 1–271) has limited solubility and was not amenable to high resolution structural analysis by NMR. The first 99 residues from the N-terminus of CaBP4 was shown previously to be unstructured, because this region was extensively cleaved in limited proteolysis studies (Shaltiel et al., 2012). Removal of the first 99 residues of CaBP4 caused marked improvement in protein solubility and did not affect target or Ca²⁺ binding. Therefore, all NMR experiments in this study were performed on an N-terminal deletion construct of mouse CaBP4 (consisting of residues 100–271), which binds functionally to Ca²⁺ and exhibits Ca²⁺-dependent binding to CaV1.4. The N-terminal deletion construct of CaBP4 (residues 100–271) with an N-terminal 6His-tag was cloned between the NdeI and BamHI restriction site of vector pET28a, using PCR primer forward 5'GGAATTCATATGCAGCAGGATGCTGCCCAAAGGAC3' and reverse 5'CGCGGATCCCTAGCCCGTAGATAGCATCATTAC3'. A plasmid pET28a vector harboring CaBP4 residues 100–271 was transformed into Rosetta 2 (DE3) cells (Novagen). The bacterial cells were pre-cultured in 30 mL LB media including antibiotics (34µg/ml chloramphenicol and 100 µg/ml kanamycin) at 37°C until the optical density at 600nm (A₆₀₀) reached 1.0. The cells were then transferred into M9 or D₂O based M9 minimal medium including antibiotics, ¹⁵NH₄Cl and D-[¹³C] glucose and grown at 37°C. The uniformly ¹⁵N-, ¹³C/¹⁵N- or ²H/¹³C/¹⁵N-labeled protein expression was induced by the addition of IPTG (at a final concentration of 0.7mM) to cells when A₆₀₀ was equal to 0.7. The bacterial cells were grown overnight after induction and harvested by centrifugation. The cell pellet was suspended and sonicated in lysis buffer containing 20 mM Tris (pH = 7.5), 5 mM imidazole, 0.5 M NaCl and 0.1 mM PMSF. The supernatant after ultracentrifugation was loaded onto Ni Sepharose column (His Trap FF, GE Healthcare) pre-equilibrated with buffer containing 20mM Tris (pH = 7.5), 5 mM imidazole and 0.5 M NaCl. The CaBP4 protein eluted from the Ni Sepharose column using an elution buffer containing 20 mM Tris (pH = 7.5), 500 mM imidazole, 0.5 M NaCl. Selected fractions containing CaBP4 were purified using a size exclusion column (Superdex 75prep, Amersham) pre-equilibrated with buffer containing 20 mM Tris (pH = 7.5), 1 mM EDTA, 1 mM DTT and 150 mM NaCl. The 6His-tag on CaBP4 was cleaved by thrombin (Novagen) treatment for 1h at room temperature. After cleavage of the 6His-tag, the final CaBP4 sample was purified using size-exclusion chromatography. The final identity and purity (> 95%) of protein was verified by SDS-PAGE.

NMR spectroscopy

All NMR experiments were performed using a Bruker Advance 800 MHz or 600 MHz spectrometer equipped with a triple resonance cryo-probe at 37°C. Samples for NMR analysis were prepared by dissolving ¹⁵N, ²H/¹⁵N/¹³C, and ¹³C/¹⁵N labeled CaBP4 (residues 100–271) (0.5 mM) in 0.3 ml of a 90/10% H₂O/D₂O solution with 10 mM

[$^2\text{H}_{11}$]Tris (pH = 7.4), 20 mM KCl, 1 mM ^2H -labeled dithiothreitol and 5 mM CaCl_2 . Backbone chemical shift assignments and side chain assignments were accomplished with ^{15}N - ^1H HSQC, HNCO, HN(CA)CO, TROSY_CACBCONH, and TROSY_HNCACB, HCCH-TOCSY, HBHACONH, ^{15}N -HSQC-TOCSY experiments as described by (Ikura et al., 1990). Stereospecific assignments of valine and leucine methyl groups were obtained by ^{13}C -edited CT-HSQC experiments performed on protein samples with directed ^{13}C labeling. NMR data were processed using NMRPipe software package and analyzed using SPARKY.

Assignments and Data Deposition

Figure 2 presents ^{15}N - ^1H HSQC spectrum of Ca^{2+} -saturated CaBP4 to illustrate representative backbone resonance assignments. NMR assignments were based on 3D heteronuclear NMR experiments performed on $^{13}\text{C}/^{15}\text{N}$ -labeled CaBP4 (residues 100–271). The first 20 residues from the amino-terminus (residues 100–120), residues 177–178 (EF2 loop), and residues 192–195 (domain linker) all exhibited weak NMR signals with random coil chemical shifts, indicative of structural disorder in these regions. The remaining residues in the core region (residues 121–271) exhibited highly dispersed NMR signals with uniform intensities, indicative of a well-defined three-dimensional protein structure. More than 95% of the backbone resonances (^1HN , ^{15}N , $^{13}\text{C}\alpha$, $^{13}\text{C}\beta$, and ^{13}CO) and ~80% of aliphatic side chain resonances were assigned for residues in structured regions, including stereospecific assignment of valine and leucine methyl resonances. Three downfield shifted amide proton resonances at ~10.5 ppm are assigned to Gly143, Gly220 and Gly257, which demonstrate that Ca^{2+} is bound functionally at EF1, EF3 and EF4, in contrast to CaBP1 that binds tightly to Ca^{2+} only at EF3 and EF4 (Li et al., 2009). CaBP4 contains glutamate at the 12-position of EF1 (E149) in contrast to CaBP1 that contains aspartate (D46), which might explain why EF1 in CaBP4 binds tightly to Ca^{2+} whereas EF1 in CaBP1 binds to Mg^{2+} (Li et al., 2009). The chemical shift assignments (^1H , ^{15}N , ^{13}C) of Ca^{2+} saturated CaBP4 have been deposited in the BioMagResBank (<http://www.bmrb.wisc.edu>) under accession number 18877.

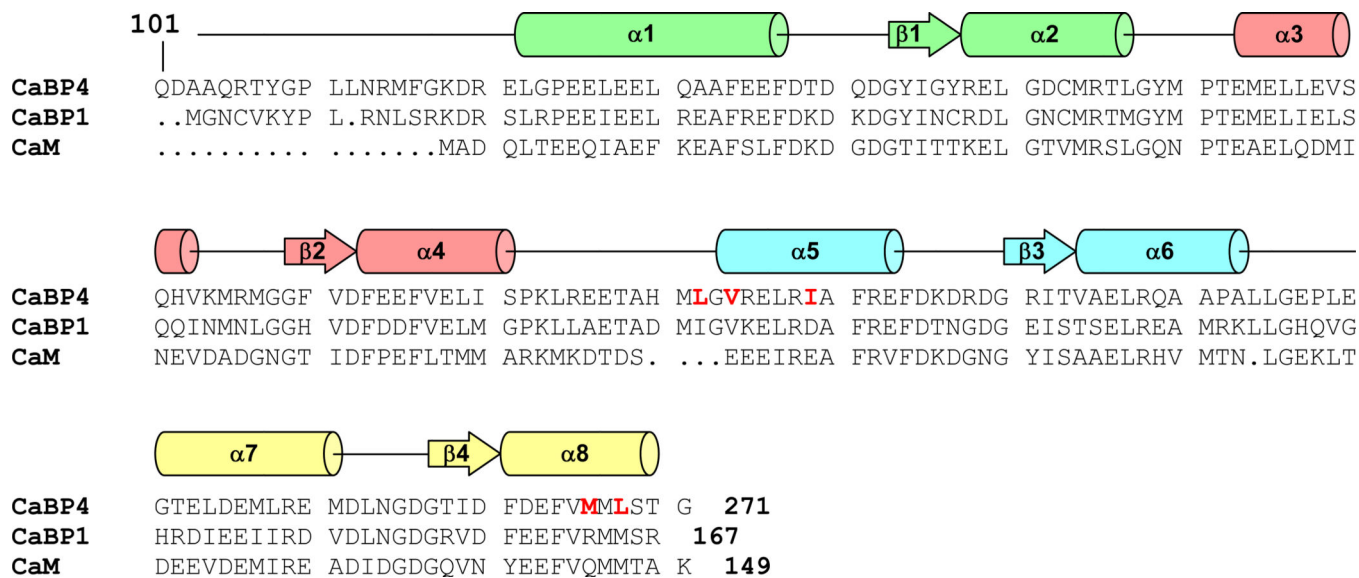
The chemical shift index (CSI) of each amino acid residue reveals a protein secondary structure in CaBP4 (Fig. 1) similar to that observed previously in CaBP1 (Li et al., 2009). CaBP4 contains 8 α -helices and two antiparallel β -sheets (α 1: 122–137; β 1: 143–146; α 2: 147–157; α 3: 163–173; β 2: 179–182; α 4: 183–191; α 5: 203–213; β 3: 220–223; α 6: 224–233; α 7: 241–251; β 4: 257–260; α 8: 261–270). A few NMR assignments reported here for Ca^{2+} saturated CaBP4 are somewhat different from the corresponding chemical shifts reported previously for CaBP1 (Li et al., 2009). The most noteworthy chemical shift differences are observed for residues in CaBP1 that are known to interact with InsP3Rs (highlighted red in Fig. 1). We suggest that these binding site residues in CaBP1 may occupy a different structural environment in CaBP4 and might explain why CaBP4 is not able to bind to InsP3Rs.

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**Figure 1.**

Alignment of the primary sequence of mouse CaBP4, CaBP1, and calmodulin. Secondary structural elements indicated schematically were derived from analysis of NMR chemical shift index (CSI) (Wishart et al., 1992). The four EF-hands (EF1, EF2, EF3 and EF4) are highlighted green, salmon, cyan, and yellow, respectively. CaBP4 residues highlighted in red exhibit markedly different backbone chemical shift values compared to those of CaBP1 (Li et al., 2009).

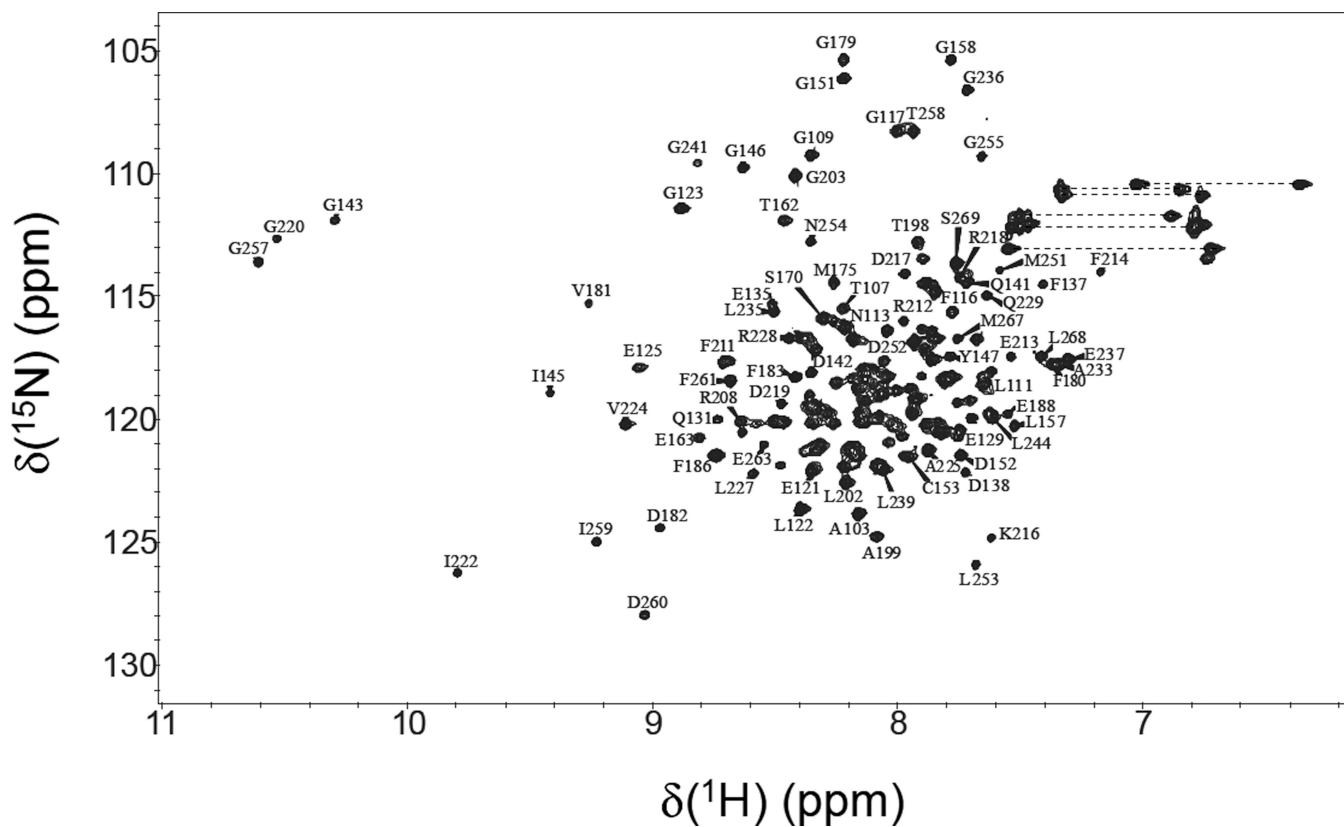


Figure 2. Two-dimensional ^{15}N - ^1H HSQC spectrum of Ca^{2+} -saturated CaBP4 recorded at 800-MHz ^1H frequency. The protein sample (0.5 mM) was uniformly labeled with nitrogen-15 and was dissolved in 0.3 ml of a 90% H_2O /10% [^2H] H_2O solution containing 10 mM [$^2\text{H}_{11}$] Tris (pH 7.4), 1 mM [$^2\text{H}_{10}$] dithiothreitol, and 5 mM CaCl_2 . Under these conditions, CaBP4 contains Ca^{2+} bound at EF1, EF3 and EF4 as detected by the three downfield NMR resonances assigned to G143, G220 and G257.