NMR resonance assignments of the catalytic domain of human serine/threonine phosphatase calcineurin in unligated and PVIVIT-peptide-bound states

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Abstract Calcineurin (Cn) is a serine/threonine phosphatase that plays pivotal roles in many physiological processes. In T cell, Cn targets the nuclear factors of activated T-cell (NFATs), transcription factors that activate cytokine genes. Elevated intracellular calcium concentration activates Cn to dephosphorylate multiple serine residues within the NFAT regulatory domain, which triggers joint nuclear translocation of NFAT and Cn. This relies on the interaction between the catalytic domain of Cn (CnA) and the conserved PxIxIT motif. Here, we present the assignment of CnA resonances in unligated form and in complex with a 14-residue peptide containing a PVIVIT sequence that was derived from affinity driven peptide selection based on the conserved PxIxIT motif of NFATs. Although a complete assignment was not possible mainly due to the paramagnetic line broadening induced by an iron in the CnA catalytic center, the assignment was extensively verified by amino-acid selective labeling of Arg, Leu, Lys, and Val, which cover one third of the CnA residues. Nevertheless, the assignments were used to determine the

structure of the CnA–PVIVIT peptide complex and provide the basis for investigation of the interactions of CnA with physiological interaction partners and small organic compounds that disrupt the Cn–NFAT interaction.

Keywords Calcineurin · Phosphatase · NFAT · T-cell

Biological context

Calcineurin (Cn) is a heterodimeric serine/threonine phosphatase that is activated by Ca^{2+} /calmodulin. The active site of calcineurin is located in the CnA subunit and is regulated by a calmodulin-like regulatory domain (CnB). CnA has one zinc and one iron ion in the catalytic core and both metals are indispensable for catalytic activity (Mondragon et al. 1997). Cn has been shown to play pivotal roles in a wide variety of biological contexts, such as regulation and development of the immune, nervous, cardiovascular and musculoskeletal systems, and in apoptosis and necrosis (Aramburu et al. 2000, 2004; Clipstone and Crabtree 1992; Hemenway and Heitman 1999; Hogan and Li 2005; Rusnak and Mertz 2000). In T-cell signaling, activated Cn dephosphorylates multiple phosphoserine residues in N-terminal regulatory regions of nuclear factors of activated T-cells (NFATs) (Jain et al. 1993). Dephosphorylation of NFAT triggers translocation of the dephosphorylated NFAT–Cn complex to the nucleus, where NFAT activates target gene expression (e.g. IL2) (Rao et al. 1997).

Cn is known as the target of immunosuppressive drugs, cyclosporin A (CsA) and tacrolimus (FK506), which block Cn's phosphatase activity by blocking the access of substrate to its catalytic site while forming stable tetrameric complexes with the intracellular proteins cyclophilin A and FKBP12, respectively (Griffith et al. 1995;

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Huai et al. 2002; Kissinger et al. 1995). However, CsA and FK506 have severe potential side effects, which might be due to the general inhibition of the Cn activity besides NFAT activation (Kiani et al. 2000). Thus the development of specific inhibitors of Cn-NFAT interaction would be of importance to develop safer immunosuppressive drugs (Roehrl et al. 2004a, b).

Two distinct Cn-binding segments that contain PxIxIT and LxVP motifs have been identified in all NFAT isoforms involved in T-cell response (i.e. NFAT1-4). Both motifs show low μM affinities toward CnA as well as the CnAB heterodimer. It has been shown recently that both motifs bind to an overlapping epitope on CnA (Gal et al. 2014) in a way that they partially compete for binding but do not fully displace each other on the CnA epitope at equimolar concentration. Thus, both segments in NFAT bind simultaneously to the overlapping epitopes on the catalytic domain. As for the PxIxIT segment, peptides that have a higher Cn binding affinity than original NFAT sequences were obtained through affinity selection from a consensus PxIxIT sequence-based combinatorial peptide library (Aramburu et al. 1999). The best peptide selected has the sequence PVIVIT at the center and exhibits a dissociation constant of 0.2 μM to CnA (Aramburu et al. 1999), compared to 2.5 μM for the wild-type sequence (Gal et al. 2014).

To obtain a detailed picture of the Cn-NFAT interaction, we had carried out mainchain assignment of CnA in unligated form and in complex with the 14-amino-acid-residue peptide with the PVIVIT sequence. The assignments have been used to determine the structure model of the CnA-PVIVIT peptide complex (Takeuchi et al. 2007b) and to characterize the interaction with a NFAT LxVP peptide (Gal et al. 2014) as well as physiological partners, and would be useful for developing new bioactive compounds that specifically target the Cn-NFAT interaction.

Materials and experiments

All chemicals were purchased from Sigma unless otherwise noted. $1\text{-}^{13}\text{C}$ amino acids were purchased from Cambridge Isotope laboratories. Celtone[®] base media were purchased from Spectra Stable Isotopes. A purified synthetic PVIVIT 14-mer peptide ($\text{H}_4\text{N-GPHPVIVITGPHEE-COOH}$) was purchased from Tufts-New England Medical Center peptide synthesis facility (Boston, MA).

Expression and purification of the catalytic domain of human calcineurin

CnA, comprising residues 2–347 with substitutions Y341S, L343A, and M347D (Aramburu et al. 1999), was produced

as a cleavable GST fusion protein in Rosetta[™] (DE3) cells. Cleavage of the fusion protein with PreScission[™] protease produces CnA (2–347) with an additional GPLG sequence at its N-terminus. CnA obtained is enzymatically active as described in our previous publication (Roehrl et al. 2004b). The $[\text{U-}^2\text{H}^1\text{C}^{15}\text{N}]$ CnA was obtained by growing the *E. Coli* in modified M9 Celtone[®] medium, which consists of 1 kg/L 99.8 % D_2O , 8.5 g/L Na_2HPO_4 , 3 g/L KH_2PO_4 , 0.5 g/L NaCl, 40 mg/L carbenicillin, 15 mg/L chloramphenicol, 1 g/L $^{15}\text{NH}_4\text{Cl}$ (99.9 % enriched), 2 g/L $^2\text{H}_6^{13}\text{C}$ -glucose (97 % enriched), 1 g/L ^2H (>97 % enriched), ^{13}C (>97 % enriched), ^{15}N (>98 % enriched) Celtone[®] Base Powder, supplemented with 2 mM MgSO_4 , 0.1 mM CaCl_2 , 10 mg/L ZnSO_4 , and 10 mg/L FeCl_3 . To obtain selectively labeled CnA, *E. coli* was grown in modified M9 Celtone medium, and desired amino acids are added after induction of protein expression at 7–10 times the amount present in the Celtone[®] media (Spectra Stable Isotopes).

After reaching an $\text{OD}_{600} = 0.6$, protein expression was induced by the addition of 1 mM IPTG at 37 °C. The cells were harvested after 36–48 h of induction for uniformly labeled samples, and after 24 h for selectively labeled samples. The harvested cells were resuspended in 40 ml of PBS with 2 mM dithiothreitol (DTT), and 0.2 mg/ml phenylmethylsulfonylfluoride (PMSF) at 4 °C. The cells were disrupted by sonication and the insoluble fraction was removed by centrifugation at 15,000g for 20 min. CnA was purified from the supernatant by Glutathione Sepharose 4 Fast Flow. CnA was eluted with 40 ml of PBS containing 2 mM DTT and 25 mM of reduced glutathione. PreScission[™] protease was added to the concentrated elution fraction, and the solution was dialyzed against 1L of PBS with 2 mM DTT for 15 h at 4 °C. The digested solution was passed through a PD-10 desalting column and Glutathione Sepharose 4 Fast Flow to remove PreScission protease and GST. The elution protein was further purified with Superdex75 size-exclusion column. A typical final yield of CnA was 2 mg/L culture.

NMR spectroscopy

All experiments were performed on a Bruker Avance 750 MHz spectrometer equipped with a cryogenic probe at 298 K. All spectra were collected using 0.4–0.6 mM protein in 10 mM sodium phosphate buffer (pH 6.8) containing 150 mM NaCl, 2 mM DTT and 90 % $\text{H}_2\text{O}/10\text{ % D}_2\text{O}$ with or without 1.2 molar excess of the non-labeled PVIVIT peptide. The backbone assignment of CnA in unligated and in complex with the PVIVIT peptide was accomplished by using standard TROSY triple resonance experiments (Ferentz and Wagner 2000). The following experiments were performed, 2D $^1\text{H}^{15}\text{N}$ -TROSY-HSQC

(Pervushin et al. 1997), 3D TROSY-HNCA (Salzmann et al. 1998), 3D TROSY-HNCOCA, 3D TROSY-HNCACB, 3D TROSY-HNCOCACB, 3D TROSY-hNcaNH (Frueh et al. 2006), and 3D $^1\text{H}^1\text{H}$ -NOESY- ^{15}N -TROSY (mixing time: 200 ms) experiments. To confirm the sequence-specific assignments, 4 types of amino-acid-specifically labeled CnA were prepared; $[\text{U-}^2\text{H}^{15}\text{N}, ^1\text{H}^{14}\text{N Arg}]$, $[\text{U-}^2\text{H}^{15}\text{N}, ^1\text{H}^{14}\text{N Lys}]$, $[\text{U-}^2\text{H}^{15}\text{N}, 1\text{-}^{13}\text{C Val}]$ and $[\text{U-}^2\text{H}^{15}\text{N}, 1\text{-}^{13}\text{C Leu}]$. Spectra were processed using XWINNMR and analyzed with Sparky (Goddard and Kneller 2006).

Assignment and data deposition

The 40 kDa CnA exhibits a well dispersed 2D $^1\text{H}^{15}\text{N}$ -TROSY-HSQC spectrum (Fig. 1), reflecting the α - β mixed structure of the protein (Griffith et al. 1995; Huai et al. 2002; Jin and Harrison 2002; Kissinger et al. 1995). About 210 resonances out of 326 expected resonances are identified in the $^1\text{H}^{15}\text{N}$ TROSY HSQC spectrum of $[\text{U-}^2\text{H}^{13}\text{C}^{15}\text{N}]$ CnA. Absence of the other resonances is primarily due to the paramagnetic broadening by the iron in the CnA catalytic center. Insufficient amide proton D/H back exchange after expression of the protein in D_2O is another reason for incomplete assignment. In an attempt to overcome this problem we prepared partially deuterated of

CnA using deuterated expression media but H_2O as solvent. This allowed for detection of ~ 20 additional resonances out of the ~ 110 missing signals (Lohr et al. 2003). Indeed, none of the assigned resonances are from nuclei within a $10 \text{ \AA}$ distance from the iron of the catalytic center (Fig. 2a). Despite several attempts we were not able to replace the catalytic iron with a diamagnetic metal. Upon titration of the PVIVIT peptide to CnA, CnA resonances exhibited slow exchange chemical shift perturbation (Takeuchi et al. 2007b) and the signal intensity is rather uniform in the PVIVIT bound state, thus the residue-specific assignments were carried out first for the CnA-PVIVIT complex and then independently for ligand-free CnA.

The impact of paramagnetic broadening is even more severe in triple resonance 3D experiments, which require longer pulse sequences. Thus, in addition to the TROSY-type triple-resonance experiments, we extensively used ^{15}N and $1\text{-}^{13}\text{C}$ selectively labeled samples (Takeuchi et al. 2007a), which enable verification of the sequence-specific assignment obtained by triple-resonance experiments. One third of the residues were selectively labeled to confirm the sequence specific assignments based on 3D triple resonance experiments (Fig. 2b). Nevertheless, the combination of TROSY-type triple-resonance experiments and selective labeling provides reliable assignments of most residues outside of the paramagnetic center. As the result, $\sim 60 \%$ of the observable HN cross peaks are assigned in

Fig. 1 Amide resonance assignment of CnA. 2D $^1\text{H}^{15}\text{N}$ -TROSY-HSQC spectrum of $[\text{U-}^2\text{H}^{15}\text{N}^{13}\text{C}]$ CnA that was recorded on Bruker Avance 750 MHz spectrometer at 298 K. Sequence specific

assignments were indicated. The spectral region that is boxed in the left panel is enlarged in the right panel. Several residues exhibited two cross peaks, reflecting the presence of multiple conformers

Fig. 2 Mapping of assigned residues of CnA. **a** The CnA surface was colored according to the availability of sequence specific assignment for the closest amide proton. The surface with and without the assignment is colored *blue* and *gray*, respectively. The iron and zinc ions in the catalytic center were drawn as orange and purple spheres.

b Mapping of assigned residues in a ribbon representation of CnA. All residues with available assignments are indicated with spheres. The residues for which the assignments were confirmed by Arg-negative, Lys-negative, $1\text{-}^{13}\text{C}$ Leu, and $1\text{-}^{13}\text{C}$ Val labeling were colored *yellow*, *green*, *red*, and *purple*, respectively

the unligated form and in complex with PVIVIT. This corresponds to $\sim 40\%$ of non-proline residues in the CnA construct used. Residues that exhibited substantial chemical shift differences between the unligated and PVIVIT-bound state are located near the exposed $\beta 14$ strand of the central CnA β sheet. This formed the basis for the NOE-based structure determination of the CnA/PVIVIT complex (Takeuchi et al. 2007b) and is consistent with the X-ray structure of the complex (Li et al. 2007).

The chemical shifts were externally referenced relative to DSS for ^1H and indirectly for ^{13}C and ^{15}N . The sequence-specific resonance assignment of the H^{N} and N^{H} atoms are deposited in BMRB data base in the absence and presence of the PVIVIT peptide with the accession numbers 19980 and 19981, respectively. The coordinates of the CnA-PVIVIT complex were deposited to the PDB with the accession number 2JOG. Note that several sidechain resonances assignments of the CnA residues in the PVIVIT binding site were established while pursuing the structure determination of the CnA/PVIVIT complex. These are, Tyr-288 $\text{H}\delta$: 6.90 ppm; $\text{H}\epsilon$: 6.55 ppm, Met-290 $\text{H}\epsilon$: 1.65 ppm, Phe-299 $\text{H}\epsilon$: 6.55 ppm; $\text{H}\zeta$: 7.36 ppm, Met-329 $\text{H}\epsilon$: 1.90 ppm, Ile-331 $\text{H}\beta$: 1.93 ppm, $\text{H}\gamma$: 0.95/1.40 ppm, $\text{H}\delta$: 1.91 ppm.

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