

10-2017

^1H , ^{15}N , and ^{13}C Chemical Shift Assignments of the Regulatory Domain of Human Calcineurin

Dinesh K. Yadav
Mississippi State University

Sri Ramya Tata
Mississippi State University

John Hunt
Mississippi State University


Erik C. Cook
University of Kentucky, erik.cook@uky.edu

Trevor P. Creamer
University of Kentucky, trevor.creamer@uky.edu

See next page for additional authors

Right click to open a feedback form in a new tab to let us know how this document benefits you.

Follow this and additional works at: https://uknowledge.uky.edu/csb_facpub

 Part of the [Biochemistry Commons](#), and the [Structural Biology Commons](#)

Repository Citation

Yadav, Dinesh K.; Tata, Sri Ramya; Hunt, John; Cook, Erik C.; Creamer, Trevor P.; and Fitzkee, Nicholas C., " ^1H , ^{15}N , and ^{13}C Chemical Shift Assignments of the Regulatory Domain of Human Calcineurin" (2017). *Center for Structural Biology Faculty Publications*. 9.
https://uknowledge.uky.edu/csb_facpub/9

This Article is brought to you for free and open access by the Center for Structural Biology at UKnowledge. It has been accepted for inclusion in Center for Structural Biology Faculty Publications by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.

Authors

Dinesh K. Yadav, Sri Ramya Tata, John Hunt, Erik C. Cook, Trevor P. Creamer, and Nicholas C. Fitzkee

 ^1H , ^{15}N , and ^{13}C Chemical Shift Assignments of the Regulatory Domain of Human Calcineurin**Notes/Citation Information**

Published in *Biomolecular NMR Assignments*, v. 11, issue 2, p. 215-219.

© Springer Science+Business Media B.V. 2017

The copyright holder has granted the permission for posting the article here.

This is a post-peer-review, pre-copyedit version of an article published in *Biomolecular NMR Assignments*. The final authenticated version is available online at: <https://doi.org/10.1007/s12104-017-9751-x>.

Digital Object Identifier (DOI)

<https://doi.org/10.1007/s12104-017-9751-x>



Published in final edited form as:

Biomol NMR Assign. 2017 October ; 11(2): 215–219. doi:10.1007/s12104-017-9751-x.

¹H, ¹⁵N, and ¹³C Chemical Shift Assignments of the Regulatory Domain of Human Calcineurin

Dinesh K. Yadav¹, Sri Ramya Tata¹, John Hunt¹, Erik C. Cook², Trevor P. Creamer², and Nicholas C. Fitzkee¹

¹Department of Chemistry, Mississippi State University

²Center for Structural Biology, Department of Molecular and Cellular Biochemistry, University of Kentucky

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 ¹³C, ¹⁵N and ¹H assignments of the RD of CaN were obtained using solution NMR spectroscopy. The backbone of RD has been assigned using a combination of ¹³C-detected CON-IPAP experiments as well as traditional HNCO, HNCA, HNCOCA and HNCACB-based 3D NMR spectroscopy. A ¹⁵N-resolved TOCSY experiment has been used to assign H α and H β chemical shifts.

Keywords

Calcineurin; Intrinsically Disordered Protein; Calmodulin Binding

Biological Context

Calcineurin (CaN), also known as protein phosphatase 2B, is a Ca²⁺ 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,

Corresponding Author: Nicholas C. Fitzkee, Department of Chemistry, Mississippi State University, Hand Lab 1115, 310 Presidents Circle, Mississippi State, MS 39762, nfitzkee@chemistry.msstate.edu, Phone: (662) 325-1288, Fax: (662) 325-1618, ORCID: 0000-0002-8993-2140.

Ethical Standards

The authors declare that all experiments performed comply with current US law.

Conflict of Interest

The authors declare no conflict of interest.

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 (Hoeffler, 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 $\mu\text{g/ml}$ ampicillin and 50 $\mu\text{g/ml}$ kanamycin) overnight at 37°C. These starter cultures were then added to 1L of M9 minimal media (containing 75 $\mu\text{g/ml}$ ampicillin and 50 $\mu\text{g/ml}$ 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 ($\text{OD}_{600} \approx 1.6$). 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, 125 $\mu\text{g/ml}$ 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 [^1H - ^{15}N]-HSQC experiments (Grzesiek and Bax 1992). Side chain ^1H 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 ^1H - ^{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 ^1H 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 $^{13}\text{C}_\alpha$ and $^{13}\text{C}_\beta$ 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, $^{13}\text{C}_\alpha$ 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 $^{13}\text{C}_\beta$ chemical shifts (Fig. 2B), which are quite random throughout the sequence. Secondary structure propensity (SSP) scores (Marsh, Singh et al. 2006) and $\delta 2\text{D}$ 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

We thank Scott Showalter and Jinfa Ying for assistance with CON spectral acquisition and data processing. We would like to thank Anthony Persechini (University of Missouri at Kansas City, Kansas City, MO) for the pETCaMI vector. Research reported in this publication was supported by the National Institutes of General Medical Sciences of the National Institutes of Health under award number R15GM113152. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

- Bai Y, Milne JS, Mayne L, Englander SW. Primary structure effects on peptide group hydrogen exchange. *Proteins: Struct Funct Bioinform.* 1993; 17:75–86.
- Bastidas M, Gibbs EB, Sahu D, Showalter SA. A primer for carbon-detected NMR applications to intrinsically disordered proteins in solution. *Conc Magn Reson A.* 2015; 44:54–66.
- Camilloni C, De Simone A, Vranken WF, Vendruscolo M. Determination of Secondary Structure Populations in Disordered States of Proteins Using Nuclear Magnetic Resonance Chemical Shifts. *Biochemistry.* 2012; 51:2224–2231. [PubMed: 22360139]
- Carlomagno T, Maurer M, Sattler M, Schwendinger MG, Glaser SJ, Griesinger C. PLUSH TACS: Homonuclear planar TACS with two-band selective shaped pulses applied to Ca, C' transfer and $\text{C}\beta, \text{Caromatic}$ correlations. *J Biomol NMR.* 1996; 8:161–170. [PubMed: 22911140]
- Conicella Alexander E, Zerze Gül H, Mittal J, Fawzi Nicolas L. ALS Mutations Disrupt Phase Separation Mediated by α -Helical Structure in the TDP-43 Low-Complexity C-Terminal Domain. *Structure.* 2016; 24:1537–1549. [PubMed: 27545621]
- Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR.* 1995; 6:277–293. [PubMed: 8520220]
- Dunlap TB, Cook EC, Rumi-Masante J, Arvin HG, Lester TE, Creamer TP. The Distal Helix in the Regulatory Domain of Calcineurin Is Important for Domain Stability and Enzyme Function. *Biochemistry.* 2013; 52:8643–8651. [PubMed: 24191726]
- Dunlap TB, Guo HF, Cook EC, Holbrook E, Rumi-Masante J, Lester TE, Colbert CL, Vander Kooi CW, Creamer TP. Stoichiometry of the calcineurin regulatory domain-calmodulin complex. *Biochemistry.* 2014; 53:5779–5790. [PubMed: 25144868]
- Ermak G, Davies KJ. Chronic high levels of the RCAN1-1 protein may promote neurodegeneration and Alzheimer disease. *Free Radical Biol Med.* 2013; 62:47–51. [PubMed: 23369757]
- Griffith JP, Kim JL, Kim EE, Sintchak MD, Thomson JA, Fitzgibbon MJ, Fleming MA, Caron PR, Hsiao K, Navia MA. X-Ray structure of calcineurin inhibited by the immunophilin immunosuppressant FKBP12-FK506 complex. *Cell.* 1995; 82:507–522. [PubMed: 7543369]
- Grzesiek S, Bax A. Improved 3D triple-resonance NMR techniques applied to a 31 kDa protein. *J Magn Reson.* 1992; 96:432–440.
- Hoeffler CA, Dey A, Sachan N, Wong H, Patterson RJ, Shelton JM, Richardson JA, Klann E, Rothermel BA. The Down syndrome critical region protein RCAN1 regulates long-term potentiation and memory via inhibition of phosphatase signaling. *J Neurosci.* 2007; 27:13161–13172. [PubMed: 18045910]
- Hubbard MJ, Klee CB. Functional domain structure of calcineurin A: mapping by limited proteolysis. *Biochemistry.* 1989; 28:1868–1874. [PubMed: 2541767]
- Jin L, Harrison SC. Crystal structure of human calcineurin complexed with cyclosporin A and human cyclophilin. *Proc Natl Acad Sci USA.* 2002; 99:13522–13526. [PubMed: 12357034]
- Kjaergaard M, Brander S, Poulsen FM. Random coil chemical shift for intrinsically disordered proteins: effects of temperature and pH. *J Biomol NMR.* 2011; 49:139–149. [PubMed: 21234644]
- Liu J, Farmer JD Jr, Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell.* 1991; 66:807–815. [PubMed: 1715244]

- Macian F. NFAT proteins: key regulators of T-cell development and function. *Nat Rev Immunol.* 2005; 5:472–484. [PubMed: 15928679]
- Manalan AS, Klee CB. Activation of calcineurin by limited proteolysis. *Proc Natl Acad Sci USA.* 1983; 80:4291–4295. [PubMed: 6576338]
- Marsh JA, Singh VK, Jia Z, Forman-Kay JD. Sensitivity of secondary structure propensities to sequence differences between α - and γ -synuclein: Implications for fibrillation. *Protein Sci.* 2006; 15:2795–2804. [PubMed: 17088319]
- Rumi-Masante J, Rusinga FI, Lester TE, Dunlap TB, Williams TD, Dunker AK, Weis DD, Creamer TP. Structural basis for activation of calcineurin by calmodulin. *J Mol Biol.* 2012; 415:307–317. [PubMed: 22100452]
- Rusnak F, Mertz P. Calcineurin: form and function. *Physiol Rev.* 2000; 80:1483–1521. [PubMed: 11015619]
- Schleucher J, Schwendinger M, Sattler M, Schmidt P, Schedletzky O, Glaser SJ, Sørensen OW, Griesinger C. A general enhancement scheme in heteronuclear multidimensional NMR employing pulsed field gradients. *J Biomol NMR.* 1994; 4:301–306. [PubMed: 8019138]
- Shen X, Li H, Ou Y, Tao W, Dong A, Kong J, Ji C, Yu S. The secondary structure of calcineurin regulatory region and conformational change induced by calcium/calmodulin binding. *J Biol Chem.* 2008; 283:11407–11413. [PubMed: 18296442]
- Stemmer PM, Klee CB. Dual calcium ion regulation of calcineurin by calmodulin and calcineurin B. *Biochemistry.* 1994; 33:6859–6866. [PubMed: 8204620]
- Takeuchi K, Sun ZY, Li S, Gal M, Wagner G. NMR resonance assignments of the catalytic domain of human serine/threonine phosphatase calcineurin in unligated and PVIVIT-peptide-bound states. *Biomol NMR Assign.* 2015; 9:201–205. [PubMed: 25209144]
- Vega RB, Bassel-Duby R, Olson EN. Control of cardiac growth and function by calcineurin signaling. *J Biol Chem.* 2003; 278:36981–36984. [PubMed: 12881512]
- Wang JH, Desai R. A brain protein and its effect on the Ca^{2+} -and protein modulator-activated cyclic nucleotide phosphodiesterase. *Biochem Biophys Res Commun.* 1976; 72:926–932. [PubMed: 186066]
- Yang SA, Klee CB. Low affinity Ca^{2+} -binding sites of calcineurin B mediate conformational changes in calcineurin A. *Biochemistry.* 2000; 39:16147–16154. [PubMed: 11123943]
- Ye Q, Feng Y, Yin Y, Faucher F, Currie MA, Rahman MN, Jin J, Li S, Wei Q, Jia Z. Structural basis of calcineurin activation by calmodulin. *Cell Signal.* 2013; 25:2661–2667. [PubMed: 24018048]
- Ye Q, Wang H, Zheng J, Wei Q, Jia Z. The complex structure of calmodulin bound to a calcineurin peptide. *Proteins: Struct Funct Bioinform.* 2008; 73:19–27.
- Zhao Y, Yang H, Meng K, Yu S. Probing the Ca^{2+} /CaM-induced secondary structural and conformational changes in calcineurin. *Int J Biol Macromol.* 2014; 64:453–457. [PubMed: 24398235]

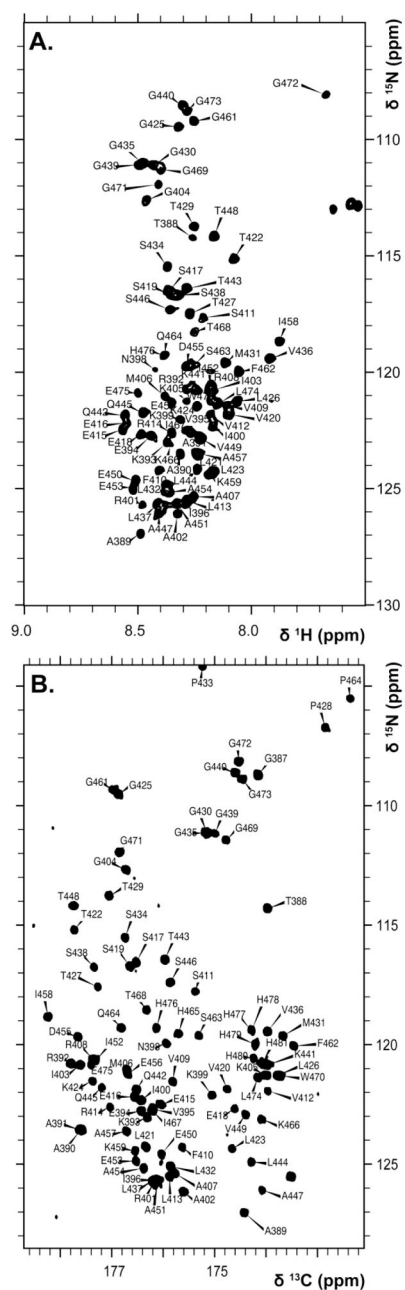


Figure 1. Assigned $[\text{}^1\text{H}-\text{}^{15}\text{N}]$ -HSQC spectrum (A) and (HACA)N(CA)CON spectrum (B) of CaN RD in solution. All three proline residues in panel B are aliased.

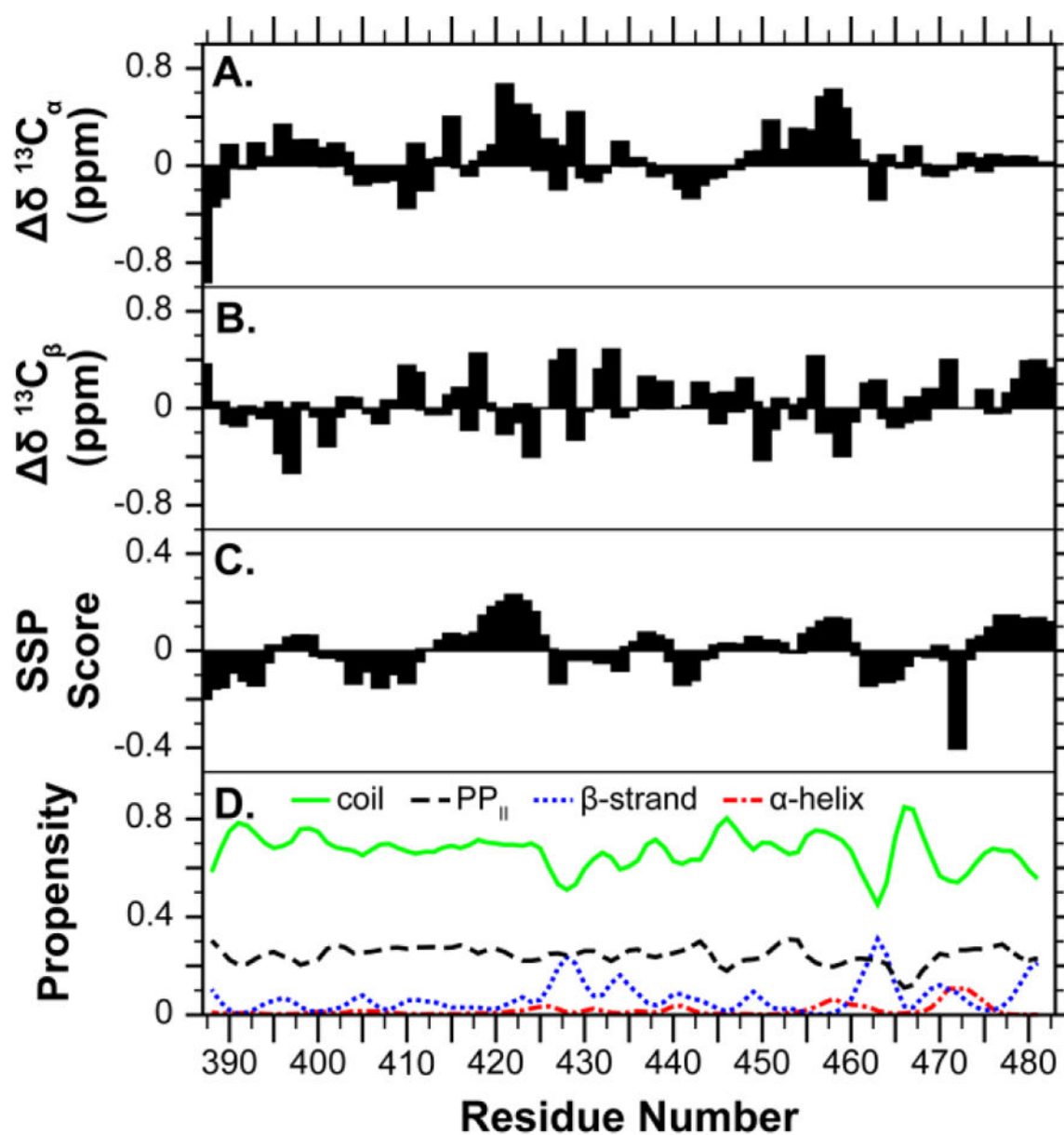


Figure 2. $^{13}\text{C}_{\alpha}$ (A) and $^{13}\text{C}_{\beta}$ (B) secondary chemical shifts for the CaN RD. Secondary $^{13}\text{C}_{\alpha}$ shifts suggest a slight propensity for helix formation between residues 417–422 and 452–460. SSP scores (C) for the RD are also weakly helical in these regions.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