



Published in final edited form as:

Biomol NMR Assign. 2011 October ; 5(2): 125–129. doi:10.1007/s12104-010-9283-0.

¹H, ¹³C and ¹⁵N NMR Assignments of the C1A and C1B Subdomains of PKC-delta

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Abstract

The Protein Kinase C family of enzymes is a group of serine/threonine kinases that play central roles in cell-cycle regulation, development and cancer. A key step in the activation of PKC is translocation to membranes and binding of membrane-associated activators including diacylglycerol (DAG). Interaction of novel and conventional isoforms of PKC with DAG and phorbol esters occurs through the two C1 regulatory domains (C1A and C1B), which exhibit distinct ligand binding selectivity that likely controls enzyme activation by different co-activators.

PKC has also been implicated in physiological responses to alcohol consumption and it has been proposed that PKC α [1, 2], PKC ϵ [3] and PKC δ [4, 5] contain specific alcohol-binding sites in their C1 domains. We are interested in understanding how ethanol affects signal transduction processes through its effects on the structure and function of the C1 domains of PKC. Here we present the ¹H, ¹⁵N and ¹³C NMR chemical shift assignments for the *Rattus norvegicus* PKC δ C1A and C1B proteins.

Keywords

Protein Kinase C Delta; C1A domain; C1B domain; diacyl glycerol; Phorbol ester binding

Biological Context

There is a significant body of evidence that alcohols can directly interact with and modulate PKC activity, including PKC δ [1–4, 6, 7]. PKC δ null mice are less sensitive to ethanol intoxication, indicating that the enzyme contributes to the behavioral response to the drug [7]. The adaptive increase of L-type calcium channels in response to chronic ethanol exposure is also contingent upon PKC δ [8], demonstrating that the enzyme may be involved in neuronal hyperexcitability that occurs during withdrawal and so contribute to a propensity for alcoholism. Understanding the basic mechanisms by which ethanol produces its cellular and physiological effects is important for developing new approaches for treating the disease.

PKC enzymes are separated into three different families based on their domain organization and cofactor requirements. Activation requires a series of phosphorylation steps and conformational changes in the protein that are induced by the binding of co-activators at

membrane surfaces. Conventional PKC (cPKC) isozymes (α , β I, β II and γ) ubiquitously bind calcium ions and phosphatidylserine at their C2 domain in addition to either DAG, or exogenously-applied phorbol esters, through their C1 domains. In contrast, novel PKC (nPKC) isozymes (δ , ϵ , η and θ) do not require calcium, but can be activated by DAG or phorbol esters alone. This calcium independence is achieved by the presence of C1 domains that have a higher affinity for ligands than those of the conventional isozymes [9].

The structures of the C1B domains from PKC α [10], and PKC γ [11] have been solved by NMR and the structure of the C1B from PKC δ domain has been solved by X-ray crystallography [12]. Further, the crystal structure of the complex between PKC δ C1B and phorbol acetate shows that ligand binding occurs between two juxtaposed hairpin loops [12]. These domains exhibit a similar overall fold that is maintained by the presence of two C3H1 zinc coordination sites. It is highly likely that the C1A domain of PKC δ will have a similar fold as the C1B domain. However, these domains bind selectively to different ligands [9, 13–18] and are potentially differentially affected by alcohols [1]. It is difficult to explain the mechanistic basis for these differences as there is no structural information available for the C1A domain and structure of the C1B domain suggest very few changes occur upon ligand binding. By solving the structure of the C1A domain and comparing the effects of alcohol on ligand binding to different C1 domains, we will be in a position to better define the mechanism of alcohol action on PKC and how this contributes to cellular changes associated with alcohol consumption. Towards this end, we report the ^1H , ^{15}N and ^{13}C NMR chain chemical shift assignments of the PKC δ C1A and C1B domains as a precursor to NMR studies to examine how alcohols influence C1:ligand/membrane interactions.

Methods

Protein Expression and Purification

PKC δ C1A and C1B proteins were cloned, expressed and purified according to standard protocols. Briefly, *Rattus norvegicus* cDNA was used as a template to separately subclone the C1A and C1B sequences between the *Bam*HI and *Sal*I restriction sites of pGEX-4T1 vector (GE Healthcare) and generate constructs containing an N-terminal GST tag for protein expression. The resulting plasmids were verified by DNA sequencing and transformed into Rosetta 2 *E. coli* cells (Novagen) for protein expression. The C1A construct contains a total of 80 amino acids (labeled 149–228) derived from PKC δ (residues 154–218) and residues from the pGEX-4T1 vector (residues 149–153 and 219–228). The C1B construct contains 82 amino acids (labeled 214–295) derived from PKC δ (residues 219–285) and residues from the pGEX-4T1 vector (residues 214–218 and 286–295). The solubility of both domains was greatly improved by inclusion of these additional residues, without which the proteins were expressed predominantly into inclusion bodies.

For NMR studies, proteins were expressed from cell cultures grown in minimal media supplemented with 2 g/L ^{13}C D-glucose (>99 atom %) and 1 g/L ^{15}N NH_4Cl (> 98 atom %). Cells were grown at 37 °C to an OD_{600} of 0.3–0.4, and protein expression induced by addition of 1 mM IPTG for 18 hours at 25°C. Cells were disrupted by treatment with lysozyme followed by sonication on ice, and the resulting solution treated with DNaseI. The clarified soluble fraction was applied to glutathione sepharose 4B resin (GE Healthcare) and

washed with 20 mM Tris-HCl pH 7.0, 150 mM NaCl, 10 μ M ZnCl₂, 1mM DTT, 25 mM CaCl₂ and 0.01% Triton X-100. Soluble protein was eluted in the same buffer following incubation with 50–100 units of thrombin at room temperature for 18 hours and purified by size exclusion chromatography using Superdex-75 resin (Amersham Biosciences) in the same buffer. Protein yields range between 8–13 milligrams per liter of cell growth, and concentrations of 300–500 μ M are readily achievable. The identity and integrity of the final proteins was confirmed by electrospray mass spectrometry and SDS-PAGE analysis.

NMR Experiments

NMR experiments were recorded using ¹⁵N and ¹³C labeled C1A and C1B protein (300–500 μ M) in 20 mM sodium phosphate at pH 6.9, 10% D₂O, 100 mM NaCl, 10 μ M ZnCl₂, 1 mM DTT and a trace amount of 2,2,-dimethyl-2-silapentane-5-sulfonic acid (DSS) as the chemical shift reference All experiments were performed at 25°C on Varian INOVA spectrometers operating at 500 MHz, 600 MHz (with cryoprobe) or 900 MHz using the Varian supplied pulse sequences. Assignments of the main-chain atoms were made using: 2D ¹H/¹⁵N-HSQC, 2D ¹H/¹³C-HSQC, 3D HNCOC, 3D HCA(CO)CANH, 3D HNCACB and 3D CBCA(CO)NH and 3D ¹H,¹⁵N-HSQC-NOESY-HSQC and ¹⁵N-edited NOESY-HSQC. While assignments of side chain ¹³C and ¹H resonances were made using 3D HCCH-TOCSY, 3D (H)C(CO)NH-TOCSY, and 2D (HB)CB(CGCD)HD and 2D (HB)CB(CGCDCE)HE for aromatic side chains. Data were processed with NMRpipe [19] and resonance assignments were determined using the Analysis program of the CCPNmr package [20].

Extent of assignments

Figure 1 presents the ¹H,¹⁵N-HSQC spectrum of PKC δ C1A and illustrates backbone amide assignments. The majority of residues from C1A domain (PKC δ residues 159–208) exhibited strong well dispersed NMR peaks, indicating that it is well structured. Several residues outside of the C1A domain (residues 149–153 and 219–228 derived from the pGEX-4T1 vector) were absent from the HSQC spectrum or are not involved in long-range NOEs, indicating that they are unstructured. For the C1A domain, backbone resonance assignments have been obtained for 98% of the H^N/N pairs (48 out of 49 non-proline residues). All ¹³Ca, ¹³CO, and 53 of 54 ¹H α backbone atoms have been assigned. In addition, 87% (201 of 232) of ¹H and 82% (120 out of 147) of ¹³C side chain atoms have been assigned. Outside of the C1A domain, 154–156, 209–214 and 218 from PKC δ and residues 152, 219, 220, 225, 226 and 228 from pGEX-4T1 have been assigned. The assignments for the PKC δ C1A have been deposited into the BioMagResBank database under accession number 17113.

The ¹H, ¹⁵N-HSQC spectrum of the C1B domain of PKC δ is presented in Figure 2 and is labeled with backbone amide assignments. Within the C1B domain (residues 231–280), most amide groups exhibit strong and disperse NMR signals, indicative of a structured protein. However, in contrast to the C1A domain, there are several amide resonances within the core C1 domain that are completely missing in the ¹H-¹⁵N-HSQC spectrum. These resonances are not observed at a range of spectrometer frequencies (500MHz–900MHz) and temperatures, implying that they are in intermediate exchange on the NMR timescale.

Because of this, backbone resonance assignments for the C1B protein have been obtained for only 76% of the H^N/N pairs (37 out of 49 non-Proline residues). In addition, 86% of ¹³C α (43 out of 50), 84% of ¹³CO (42 out of 50), and 85% of ¹H α (46 out of 54) backbone atoms have been assigned. While assignments have been obtained for 68% (159 out of 235) of the ¹H atoms and 67% (96 out of 144) of the ¹³C side chain atoms. The missing assignments almost completely coincide with residues for which ¹HN chemical shifts could not be obtained due to intermediate exchange. Outside of the C1B domain, residues 219–229, 281–285 derived from PKC δ and residues 217, 218 and 286–295 derived from the pGEX-4T1 have also been assigned. The ¹H, ¹³C and ¹⁵N assignments for PKC δ C1B have been deposited into the BioMagResBank database under accession number 17112.

The missing assignments within the C1B domain are for residues 237–239, 241–242, 251–254, 256–257 and 258 and these residues are mostly located in the loops of that form the ligand binding site in C1B (Figure 4). The addition of ligands including phorbol acetate, phorbol esters, or DAG does not lead to any improvement in the NMR spectra, and these residues are still not observable, suggesting that there is a significant degree of conformational exchange in these regions of the protein.

Discussion

In this work, we have determined the backbone and side chain resonance assignments for the C1A and C1B regulatory domains of PKC δ . These domains are important in binding to regulatory co-factors and have been implicated in the potential alcohol sensitivity of this protein. This study has revealed significant differences in the intrinsic dynamics of the C1A domains compared to the C1B domain. There are a number of residues in the C1B domain that are in intermediate exchange on the NMR timescale and are not observable in the ¹H-¹⁵N HSQC spectrum of this domain, indicating that they are significantly more dynamic than the same region in the C1A domain (Figure 4) [12]. The majority of these resonances belong to amino acids residing in the two proximal loops that bind phorbol acetate. In contrast, the corresponding residues in the C1A domain of PKC δ are all observable suggesting this region is significantly more ordered. This difference in dynamics may account for previously observed differences in ligand binding specificity of these domains.

The crystal structures of the C1B domain suggest that there are very few if any changes in the structure of the protein upon binding phorbol acetate, and that the ligand binding site is well structured in the absence of ligand [12]. Our studies suggest that this is not the case in solution. However, this does not appear to be a result of any significant difference in the overall solution structure compared to the crystal structure. A number of medium ($i > 2$) and long range ($i > 4$) HN to HN NOEs that are predicted from the crystal structure are clearly observed in the HSQC-NOESY-HSQC and NOESY-HSQC spectra which supports the conclusion that the overall structure observed in the crystal is essentially maintained in solution. We are currently investigating how alcohols affect the structure and dynamics of these domains, and how alcohol influences binding of ligands to these domains.

Acknowledgements

We thank Dr. Geoff Armstrong for his assistance with operation of the 900 MHz spectrometer. This work was supported by NIH/NIAAA grant AA013618-06A1 to DNMJ. The operation of the NMR facilities is supported by the University of Colorado Cancer Center Support Grant (NIH 5P30-CA046934) and operation of the 900 MHz NMR by NIH grant 5P41-GM068928.

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| C1A | 149 | GSPEFH | YIKN | HEFIATFFGQ | PTFCSVCKEF | VWGLNKQGYK | 188 |
| C1B | 214 | GSPEFIF | QKERFNIDMP | HRFKVYNYMS | PTFCDHCGSL | LWGLVKQGLK | 260 |
| | | | | * * : **** * : :*** ** * | | | |
| C1A | 189 | CRQCNAAIHK | KCIDKIIGRC | TGTATNSRDT | VDSSGRIVTD | | 228 |
| C1B | 261 | CEDCGMNVHH | KCREKVANLC | GINQKVDSSG | RIVTD | | 295 |
| | | * :* | :* : ** :* | | | | |

Figure 1.
 Alignment of protein constructs used in this study. Amino acids derived from the wild-type C1A and C1B domains are in black and residues derived from the pGEX-4T1 vector are in grey. The extents of the conserved C1 domains are indicated in the boxed region, identical residues are indicated with an asterisk (*) and strongly conserved residues are indicated with a colon (:). Alignment and analysis based on CLUSTAL W algorithm [21].

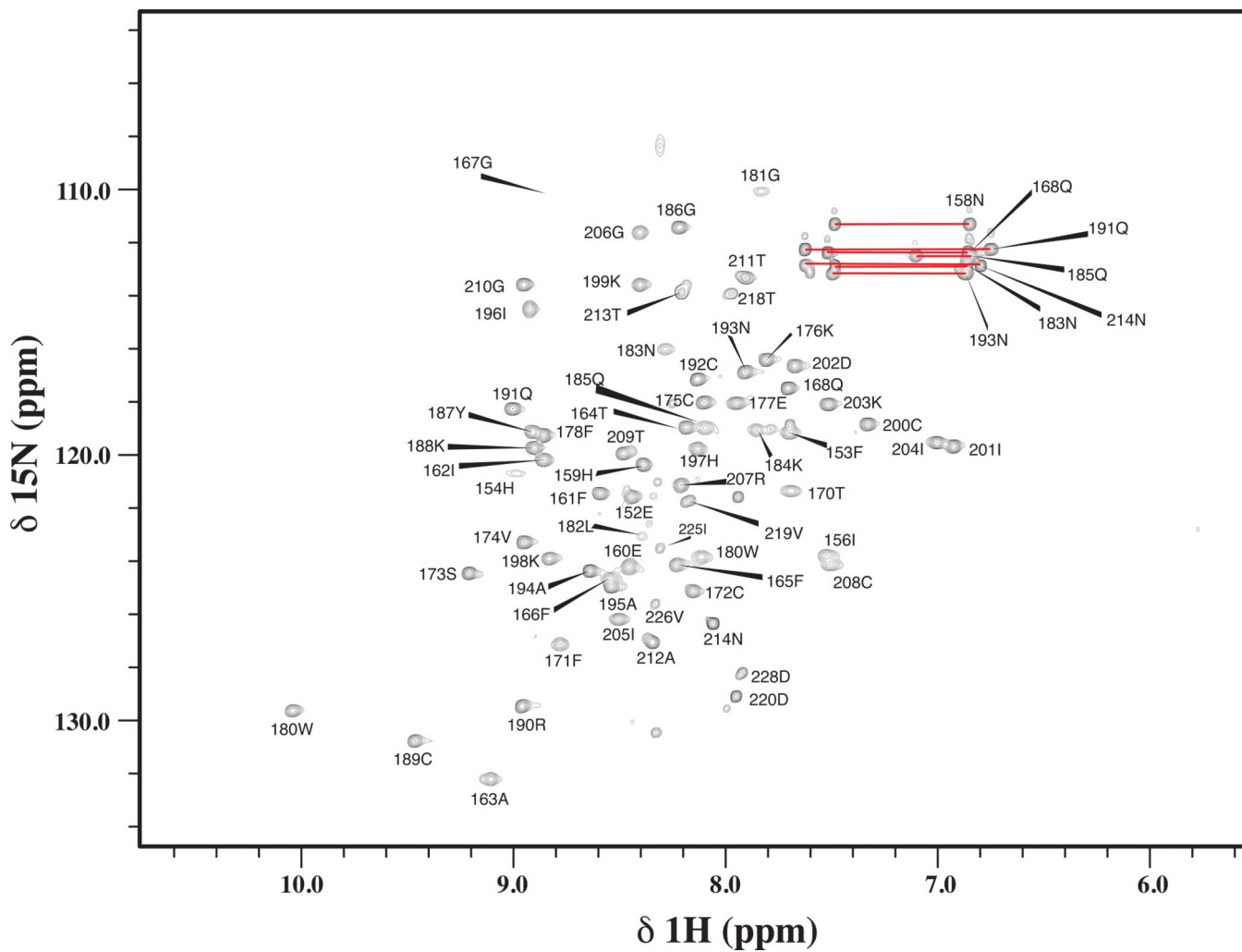


Figure 2. ^1H , ^{15}N -HSQC spectrum of PKC δ C1A recorded at 600 MHz. Each peak labeled with the chemical shift assignments. The side chain amides for Asn and Gln residues are linked by horizontal bars.

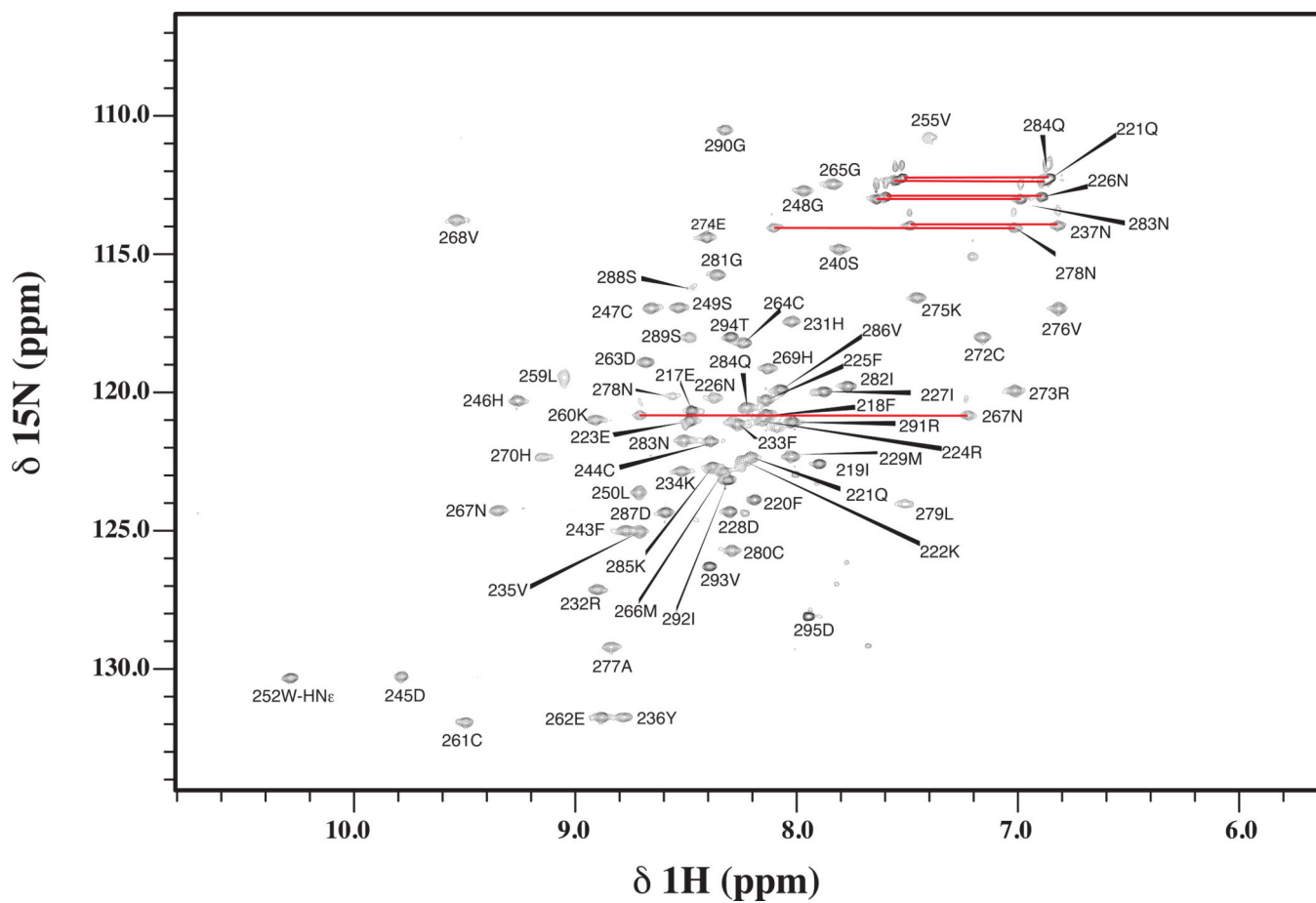


Figure 3. ^1H , ^{15}N -HSQC spectrum of PKC δ C1B recorded at 600 MHz. Each observable amide is labeled with its assignments within this construct. The side chain amides for Asn and Gln residues are linked by horizontal bars.

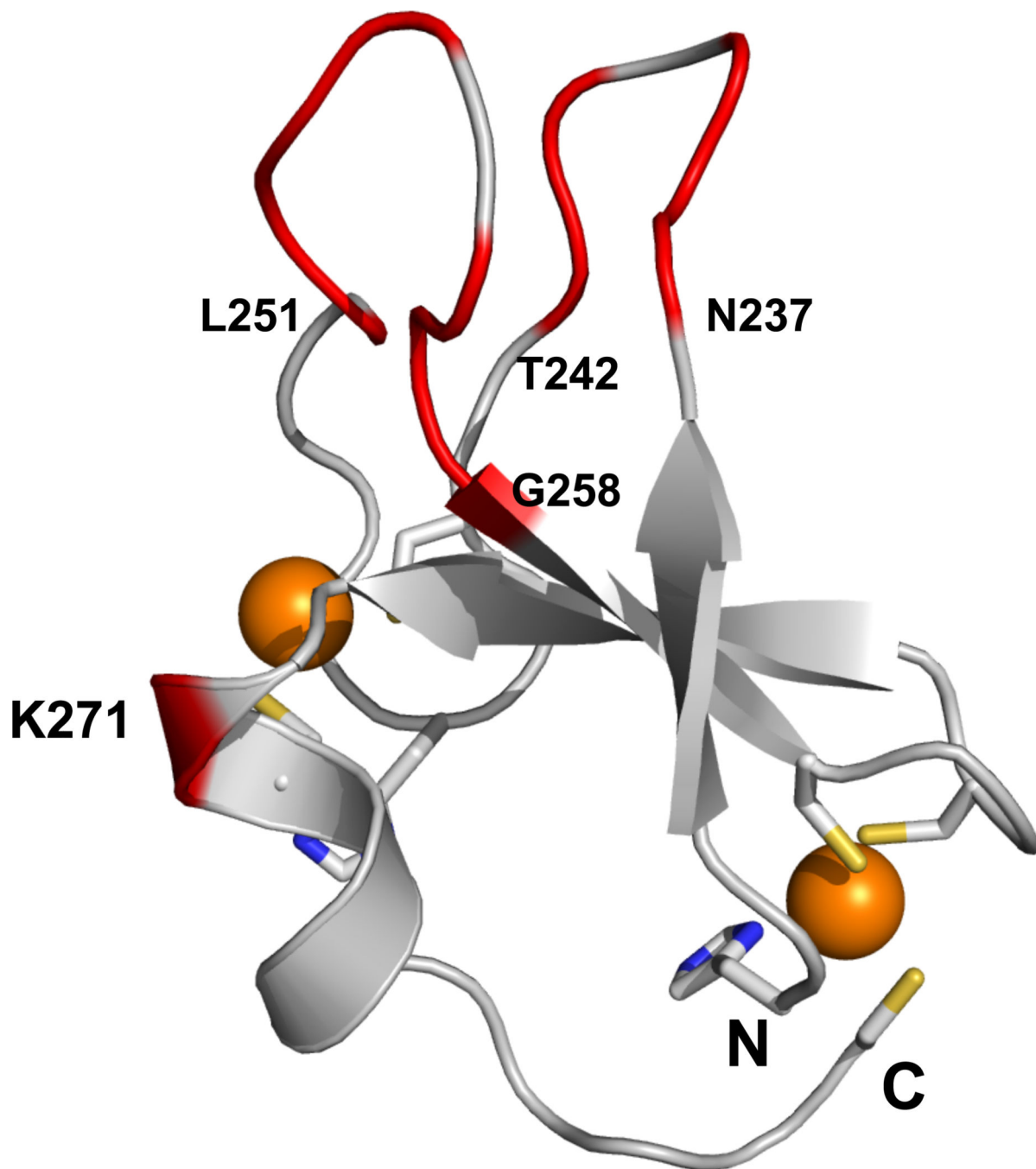


Figure 4. Ribbon diagram of the core C1B domain of PKC δ (based on PDB ID 1PTQ [12]). The location of the two zinc binding sites are shown in orange. Residues that cannot be observed in either $^1\text{H},^{15}\text{N}$ -HSQC or $^1\text{H},^{13}\text{C}$ -HSQC are colored red. This includes almost all residues in the phorbol acetate binding loops between residues N237-T242 and L251-G258. This directly contrast with the C1A domain, where all these residues can be observed