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Backbone NMR resonance assignment of the catalytic subunit of cAMP-dependent protein kinase A in complex with AMP-PNP

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Abstract

The catalytic subunit of protein kinase A is involved with a number of signal transduction pathways and has been used as a benchmark to study the structural biology and biochemistry for the entire kinase family of enzymes. Here, we report the backbone assignment of the intact 41 kDa catalytic subunit bound to AMP-PNP.

Keywords

Protein kinase A; NMR; protein backbone resonance assignments; dual amino acid selective labeling; CCLS-HSQC

Biological context

Protein kinase mediated phosphorylation is a key process for cellular signal transduction. Approximately 30% of all proteins in the human genome undergo reversible phosphorylation (Cohen 2001). As a consequence, abnormal protein kinase activity has been recognized as one of the main causes for diseases such as cancer, diabetes, rheumatoid arthritis, and some types of dilated cardiomyopathy. In fact, drugs such as rapamycin and flavopiridol have been shown to target protein kinase activity (Cohen 2001).

Since Protein Kinase A (EC: 2.7.11.11) is the most conserved protein kinase, it has served as the prototype to study the kinase family (Johnson et al. 2001). The catalytic subunit of PKA (PKA-C) shares the domain organization of all of the kinase catalytic domains, with a *bean-like* arrangement of two lobes to form an active site cleft. The small lobe is primarily associated

with binding and positioning the nucleotide ligand, while the large lobe binds a protein substrate and provides a docking surface for the regulatory subunits (Johnson et al 2001). Highly conserved motifs within PKA-C include: the C-helix (located in the small lobe) and a series of dynamic loops responsible for catalysis and positioned at the fringe between the small and the large lobe (the peptide positioning loop, the activation loop, the Mg^{2+}-positioning loop, Glycine-rich loop, and DFG loop). Mutations within these motifs have been shown to cause large scale changes in kinase activity and/or proper folding of the enzyme (Johnson et al. 2001).

While a wealth of structural data is available for PKA-C from x-ray crystallography (Johnson et al. 2001), little is known about the atomic level detail of its dynamics or interaction with membrane proteins. Due to their dynamic nature, intact protein kinase domains are notoriously resistant to NMR analysis and a handful of NMR studies are now available. Our initial NMR investigation on PKA-C demonstrated the existence of a positive allosteric cooperativity driven in part by a dynamic sampling of conformational states. In order to fully analyze the dynamic transitions of PKA-C by NMR, we have carried out the backbone resonance assignment of the enzyme bound to AMP-PNP (5′-adenylyl-β,γ-imidodiphosphate) using ^{2}H, ^{13}C, and ^{15}N labeled protein and extensive selective labeling. To date, we reached ~80% of the total resonances, leaving only regions in helices D and E (both located at the stable core of the enzyme) unassigned. Taken together with the limited NMR characterizations of the enzymatic family of kinases, such as Abl kinase (Vajpai et al. 2008), this represents a step towards studying the dynamical features and molecular interactions by NMR.

Methods and experiments

PKA-C expression and purification was carried out based on the protocols established by Taylor and co-workers (Yonemoto et al. 1991). Based on our screening of optimal $^{2}H_2O$ concentration for expression, M9 minimal media supplemented with $^{15}NH_4Cl$ and D-glucose-$^{13}C_6$-$^{2}H_7$ in 80% $^{2}H_2O$ was used to produce perdeuterated enzyme for triple resonance experiments. Deuterium incorporation was greater than 95% based on mass spectrometry (see Supplementary Materials Figure S1 and Table S1). Extensive selective amino acid labeling was utilized for the identification of 15 residue types in $^{1}H/^{15}N$ TROSY-HSQC spectra: Ala, Val, Leu, Ile, Lys, Arg, Thr, Ser, Gly, His, Phe, Tyr, Trp, Met, and Asn. Introduction of specific 1-^{13}C and ^{15}N labeled amino acids for dual amino acid labeling allowed the assignment of the following unique $^{13}C'$-^{15}N linked dipeptides in the primary sequences: LL (3 occurrences), LI (3 occurrences), FG (2 occurrences), and GF (1 occurrence). Cells were grown at 30 °C until reaching an OD of 1.2, at which the temperature was reduced to 24 °C and protein overexpression was induced by the addition of 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Typically, expression was allowed to proceed for 12 hours (deuterated media) or 5 hours (non-deuterated media). Cells were harvested by centrifugation at 7,000 *g* for 20 minutes at 4 °C and flash frozen in N_2 (*l*) prior to purification. Cell pellets were resuspended in lysis buffer containing 100 mM MES (pH 6.5), 5 mM β-mercaptoethanol, and 0.15 mg/ml lysozyme; and lysed on ice by sonication for 10 minutes. Cell debris were cleared by centrifugation at 75,000 *g* and PKA-C was isolated by binding the supernatant batch-wise to P11 phosphocellulose resin and eluting over a gradient of 0-250 mM K_2HPO_4 dissolved in lysis buffer. Fractions containing purified enzyme were pooled and dialyzed against 20 mM K_2HPO_4 (pH 6.5), 25 mM KCl, and 5 mM β-mercaptoethanol (buffer A). Since recombinant PKA-C is found to have three phosphoisoforms (containing either 2, 3, or 4 phosphoserine or phosphothreonine residues), isoforms were separated by cation exchange chromatography on a Mono-S column using a gradient of 0-30% KCl dissolved in buffer A. All NMR experiments were performed on the isoform containing three phosphates.

Triple resonance NMR experiments were performed on a sample containing 0.28 mM PKA-C, while all other samples contained 0.5-1.0 mM total enzyme. Samples were dissolved in 180 mM KCl, 20 mM KH_2PO_4 (pH 6.5), 1 mM NaN_3, 20 mM DTT, 15 mM $MgCl_2$, 5% ^2H_2O, and 15 mM of the non-hydrolyzable nucleotide analogue, AMP-PNP. All experiments were performed at 300 K on Varian Inova 800 or 600 MHz NMR spectrometers equipped with triple resonance cryogenic probes with z-axis pulse field gradients. Backbone assignments were based on TROSY versions of HSQC, HNCA, HN(CO)CA, HNCAB, HNCO, HN(CA)CO, a $^1H/^1H$ NOESY-HSQC (τ_{mix} = 100 ms) (Grzesiek and Bax 1992) as well as a non-TROSY version of a nitrogen-edited HSQC-NOESY-HSQC (τ_{mix} = 100 ms) (Grzesiek et al. 1995). Due to the detection of broad resonances from real-time ^{13}C evolution, isotope effects of ^{13}C resonances from attached ^1H and ^2H were not observed. Unique resonances resulting from dual selective amino acid labeling were observed by using either a 2D HNCO or a CCLS-HSQC sequence (Tonelli et al. 2007). All data were processed using the software NMRPipe (Delaglio et al. 1995) and visualized with SPARKY (Goddard and Kneller 2006).

Extent of assignments and data deposition

A $^1H/^{15}N$-TROSY-HSQC of perdeuterated PKA-C is shown in Figure 1. Backbone resonance assignments have been achieved for 80% of the non-proline amide groups, ~80% of the C^α and C' resonances and 47% of the C^β resonances. Despite a handful of missing assignments which are located in two long helices in the stable core of the enzyme (Johnson et al. 2001), all of the highly conserved regions have been assigned and include the C-helix, peptide positioning, activation, Mg^{2+}-positioning, glycine-rich, and DFG–loops. These resonance assignments compare well with the previous partial assignment (~55%) of the apo-enzyme (Langer et al. 2004). It should be noted that higher signal-to-noise was observed when PKA-C was bound to AMP-PNP relative to the apo form of the enzyme, although the glycine and two phenylalanine resonances in the ^{184}DFGF^{187} sequence were broadened beyond detection. These were assigned using the apo enzyme with a CCLS-HSQC experiment as has been noted elsewhere (Tonelli et al. 2007). The remaining missing assignments are largely located in two long helices (helices D and E), and are due to missing C^β resonances in the HNCACB spectra and severe overlap for C^α or $15N^\alpha$ resonances. A chemical shift index of assigned C^α resonances is provided in Supplementary Materials as Figure S2.

The chemical shift values for ^1H, ^{13}C, and ^{15}N resonances of PKA-C have been deposited at the BioMagResBank (http://www.bmrb.wisc.edu) under accession number 15985.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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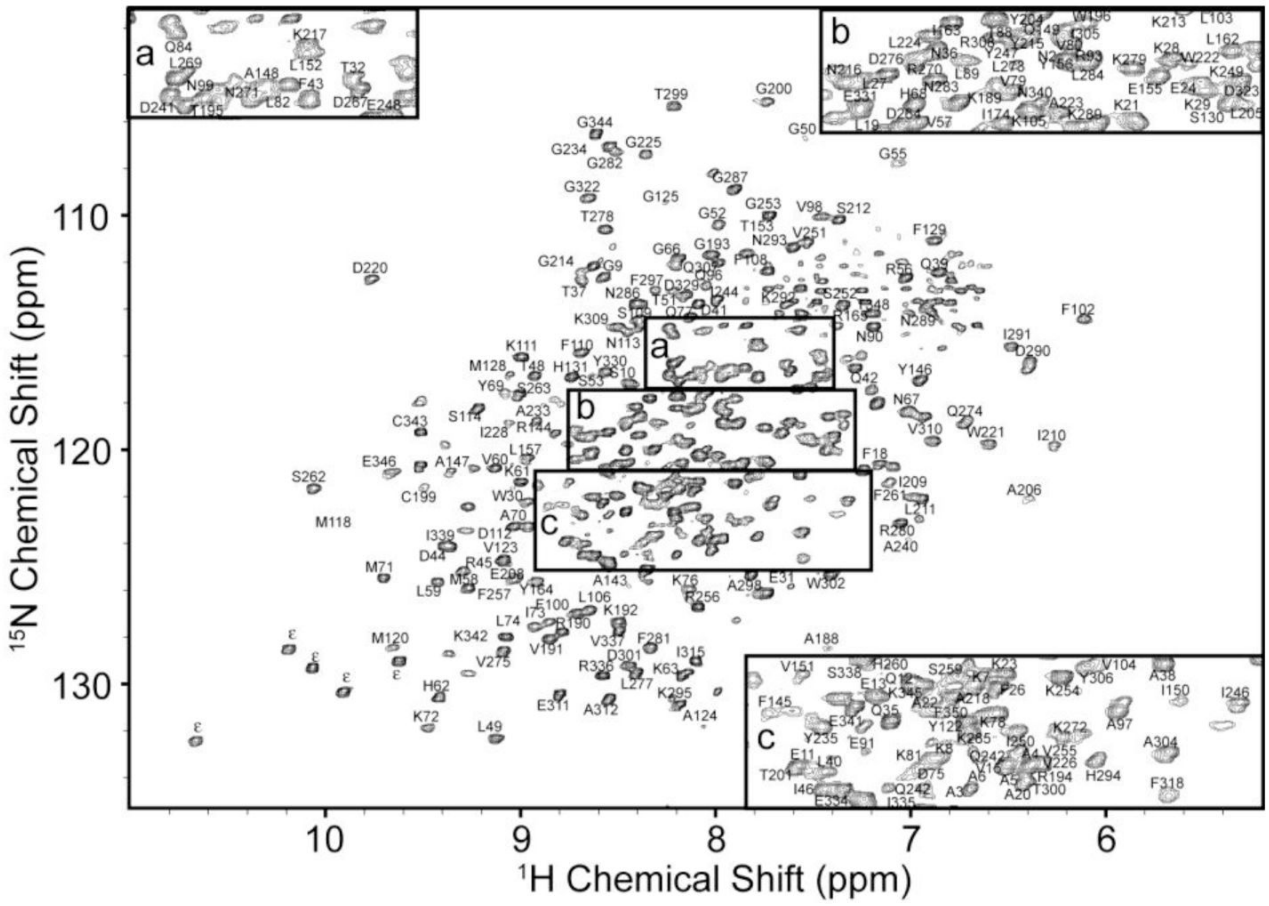

Fig. 1.

$^{1}H/^{15}N$-TROSY-HSQC spectrum of perdeuterated PKA-C complexed to AMP-PNP in 20 mM KH_2PO_4 (pH 6.5), 180 mM KCl, 20 mM DTT, and 1 mM DTT collected at 300 K on a Varian Inova 800 MHz NMR spectrometer. Assignment information is placed next to peaks and subpanels a, b, and c are provided for clarity. Indolic nitrogen resonances of tryptophan are denoted with the symbol ε.