Journal of Biomolecular NMR, **18:** 367–368, 2000.
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Letter to the Editor: ^{1}H, ^{15}N, and ^{13}C NMR resonance assignments for the Eps15 homology domain of Reps1

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Received 1 August 2000; Accepted 25 September 2000

Key words: calcium binding protein, EF hand, EH domain, endocytosis, Ral signal transduction

Biological context

The recently described Eps15 Homology (EH) domain is a protein recognition domain with a preference for binding Asp-Pro-Phe (NPF) amino acid sequences (Salcini et al., 1997). The EH domain was first identified from three repeated motifs in Eps15, a substrate for the tyrosine kinase activity of the epidermal growth factor (EGF) receptor. Since then, the motif has been found in a number of proteins from yeast to mammals (Marsh and McMahon, 1999; Mayer, 1999). An EH domain has been recently identified in the **R**alBP associated **EPS** homology domain protein **1** (Reps1) (Yamaguchi et al., 1997). The primary sequence data imply that Reps1 EH domain has two EF-hand like structures, with the second motif functional for binding calcium. Reps1 also binds to RalBP1, a GTPase-activating protein for CDC42 and Rac GTPases. RalBP1 is a downstream target of the Ral GTPase, which itself has been implicated in the regulation of clathrin-mediated endocytosis. Another RalBP1 binding protein, POB1, also contains an EH domain and is also involved in endocytosis (Yamaguchi et al., 1997; Nakashima et al., 1999). Here, we describe the strategies used to obtain NMR resonance assignments of the Reps1 EH domain.

Methods and results

The EH domain (227–318) from the murine Reps1 was expressed as a glutathione S-transferase (GST) fusion protein in the pGEX-2T vector (Pharmacia) in BL21 (pLysS), and is re-numbered here as residues 6–97. The cloning procedure resulted in the addition of five amino acids (Ala-Ser-Val-Gly-Ser) to the N-terminus. Rich medium (Luria-Bertani; LB) was used in preparing unlabeled protein. Uniformly ^{15}N- and/or ^{13}C-labeled proteins were prepared by growing cells in M9 minimal media with $^{15}NH_4Cl$ (1 g/L) and/or [^{13}C]-glucose (4 g/L) as the sole nitrogen and carbon sources, respectively.

The EH domain was purified by Glutathione Sepharose resin according to the manufacturer's protocol followed by thrombin cleavage and Benzamidine Sepharose column chromatography (Pharmacia) to eliminate thrombin. The optimal salt concentration and pH for the sample were determined using microdialysis cells (Bagby et al., 1997). Solutions with more than 100 mM NaCl, or pH values less than 5.8, resulted in precipitation. NMR samples contained 1.0 to 1.5 mM protein in a buffer of 10 mM deuterated imidazole (pH 6.7), 10 mM NaCl, 2 mM $CaCl_2$, 5 mM DTT, and 0.01% NaN_3.

Spectra were recorded on Bruker AMX 500 and Avance 600 spectrometers at 30 °C. Proton chemical shifts were referenced with respect to water at 4.71 ppm at 30 °C and the ^{15}N and ^{13}C chemical shifts were referenced using the method of gyromagnetic ratios (Wishart et al., 1995). TOCSY mixing times were 27 and 49 ms and the NOESY mixing time was 100 ms. Forward linear prediction was used to improve spectral resolution in the ^{15}N and ^{13}C dimensions of 3D data. Spectra were processed and analyzed on a Silicon Graphics workstation using the FELIX 98.0 software package. Of the expected 90 backbone resonances (97 minus the six proline residues and the N-terminus), 85 were observed in a 2D ^{1}H/^{15}N HSQC spectrum (Figure 1). Backbone resonances were assigned by a sequential assignment strategy from 2D

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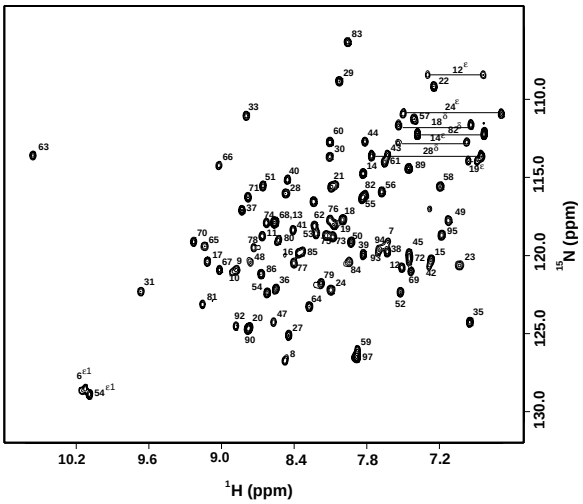

Figure 1. Two-dimensional ^{1}H-^{15}N HSQC spectrum of 1.5 mM Reps1 EH domain at 30 °C and pH 6.7, collected at 500 MHz. Peaks from the side chains of asparagines and glutamines are linked with horizontal lines.

TOCSY, NOESY, and DQF-COSY spectra and from 3D ^{15}N-separated TOCSY- and NOESY-HSQC spectra. ^{1}H/^{15}N HSQC spectra of ^{15}N-Leu or ^{15}N-Phe selectively labeled samples resolved ambiguities on some backbone resonances. HNCA, HN(CO)CA, CBCANH, CBCA(CO)NH experiments linked residues in regions that showed weak or overlapped NOE peaks, particularly in the proline-rich C-terminal region of the domain. Assignments of the non-aromatic side chains were obtained using 2D TOCSY, 3D ^{15}N-separated TOCSY, and 3D HCCH-TOCSY experiments. Carbon chemical shifts were assigned from ^{1}H/^{13}C constant time HSQC and HCCH-TOCSY experiments and the triple resonance experiments. Aromatic spin systems were assigned using 2D DQF-COSY, TOCSY, and NOESY experiments collected in 99.9% D_2O solution.

Extent of assignments and data deposition

All ^{1}H, ^{15}N, and ^{13}C backbone resonances were assigned except for the five extra amino acids in the N-terminal extension, Trp^6 HN, and the HN of residue Glu^{88}. Resonances were absent from the N-terminal residues, probably because of rapid motion and rapid hydrogen exchange. Comparison of the Hα and ^{13}Cα chemical shifts to those of the corresponding amino acids in a random coil indicates that residues 10–22, 33–42, 50–58, and 68–82 are in α helices, and that residues 29–32 and 64–66 comprise a β sheet. The secondary structure elements correspond to those in previously published structures of other EH domains that bind calcium using the second EF-hand motif (de Beer et al., 1998; Koshiba et al., 1999). Side chain proton assignments were >99% complete for residues 6–97. All aromatic protons were assigned with the exception of the ϵ and ζ ^{1}H resonances of Phe^{58} and the ζ ^{1}H resonance of Phe^{70}. Forty-three out of 73 vicinal β protons and all methyl groups were stereospecifically assigned except those of Leu^{27} and Leu^{86} because of resonance overlap. Residues 7 through 9 and 77 through 84 showed a small doubling of resonances in approximately a 60 to 40 ratio. The largest doublets were observed for residues 7, 8, and 78 to 81 (0.06–0.08 ppm in ^{1}H, 0.2–0.3 ppm in ^{15}N). The source of the micro-heterogeneity is not known. It is unlikely to originate from cis-trans isomerization about the bond preceding proline, because the nearest proline is residue 87, and both major and minor forms showed the same NOE cross peak patterns. The assignments for the major form of the EH domain from Reps1 at pH 6.7 and 30 °C have been deposited in the BioMagResBank (accession number 4788).

Acknowledgements

This research was supported by the American Cancer Society (J.D.B.) and the National Institutes of Health (L.A.F.).

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