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Letter to the Editor: ^{1}H, ^{13}C and ^{15}N backbone assignment and secondary structure of the 19 kDa diadenosine 5′, 5‴-P^{1}, P^{4}-tetraphosphate hydrolase from *Lupinus angustifolius* L.

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Biological context

Diadenosine polyphosphates (Ap_nA) have been proposed as intracellular and extracellular signaling molecules in cells. Intracellular pools of Ap_nA have been linked to a variety of biological processes, including metabolic responses to stress and the regulation of proliferation, while extracellular functions of these nucleotides include neurotransmission and vasodilation (Ogilvie et al., 1996). Diadenosine 5′, 5‴-P^{1}, P^{4}-tetraphosphate (Ap_4A), one of the major diadenosine polyphosphates, is found in almost all cells and competitively inhibits ADP-induced platelet aggregation associated with blood clotting. Analogues of Ap_4A have therefore been proposed as potential therapeutic agents and there has been a consequent interest in purifying and characterizing the enzymes that catabolize Ap_4A and its analogues. These include members of the MutT motif protein family of nucleotide phosphohydrolases, also named 'nudix' hydrolases. Amongst the nudix hydrolases for which DNA sequences have recently been obtained are the asymmetric Ap_4A hydrolases from lupin (Maksel et al., 1998) and barley (Churin et al., 1998). Of significant interest, the sequences of these hydrolase genes are homologous to a gene (*ial*A) isolated from the human pathogen *Bartonella bacilliformis* (Cartwright et al., 1999). Here we report NMR resonance assignments of Ap_4A hydrolase from lupin and compare the secondary structure analysis by CSI to the only nudix hydrolase for which a 3D structure has been obtained, the MutT enzyme from *E. coli* (Abeygunawardana et al., 1995).

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Methods and results

The gene encoding residues 1–160 of Ap_4A hydrolase was subcloned from the previously identified cDNA (Maksel et al., 1998) into the pGEX-6-P3 vector (Pharmacia) and expressed in *E. coli* BL21(DE3) in 1 L of minimal media and a 2 L fermentor at 37 °C, following the method of Cai et al. (1998). The culture was divided into four ∼250 ml portions, and the cell pellets were collected and frozen at −20 °C. Single 250 ml pellets were thawed on ice, and lysed in 20 ml of B-PER (Pierce). Cell debris was removed by centrifugation and the supernatant was passed through a 15 ml glutathione Sepharose 4B column (Pharmacia). The GST fusion product was eluted and cleaved overnight with PreScission protease (Pharmacia) at pH 8.0 and 4 °C (50 mM Tris-HCl, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA). It was further purified on glutathione Sepharose and a Mono-Q column, eluted with a 0–250 mM NaCl gradient in 25 mM Tris-HCl at pH 8.0 and 4 °C. The final cleaved and purified protein has additional residues (Gly,Pro,Leu,Gly,Ser) at its N-terminus, due to the multiple cloning site of the pGEX-6-P3 vector and PreScission protease recognition site.

Samples of Ap_4A hydrolase (0.55 ml, 0.7 to 1.2 mM) were prepared for NMR spectroscopy in a 50 mM phosphate buffer, pH 6.5, 1.5 mM dithiothreitol, 3 mM EDTA, 0.02% sodium azide and either 100% $^{2}H_2O$ or 90% H_2O/10% $^{2}H_2O$. Using a Varian 600 Inova operating at 25 °C the following spectra were acquired: 2D ^{15}N HSQC, 3D CBCANH, CBCA(CO)NH, C(CO)NH-TOCSY (18.2 ms mixing time), HNCO, HCACO, (HCA)CO(CA)NH, HCCH-

TOCSY (15.6 ms mixing time), H(CCO)-TOCSY (18.2 ms mixing time), ^{15}N NOESY-HSQC (100 ms mixing time), and ^{13}C NOESY-HSQC (100 ms mixing time) (Cavanagh et al., 1996). Aromatic assignments were established by 2D (Hβ)Cβ(CγCδ)Hδ and (Hβ)Cβ(CγCδCε)Hε and 3D ^{13}C NOESY-HSQC spectra. All spectra except the HCCH-TOCSY and NOESY-HSQC spectra were acquired with gradient selection and sensitivity enhancement. Data were processed with NMRPipe (Delaglio et al., 1995) and analyzed with XEASY (Bartels et al., 1995). ^{1}H chemical shifts were referenced to DSS at 0 ppm, and ^{13}C and ^{15}N chemical shifts were calculated from the ^{1}H spectrometer frequency.

Extent of assignments and data deposition

The assignment of the backbone segments (N, HN, C′, Cα, Hα, Cβ) is complete for all residues except Pro-80 and -81 and Lys-82, and thus all ^{15}NH correlations, except Lys-82, have been assigned (Figure 1A). Most sequence specific assignments were obtained by the combination of the CBCANH and CBCA(CO)NH experiments. These data were confirmed, and ambiguities resolved through the combination of HNCO and (HCA)CO(CA)NH experiments. To further confirm and extend these assignments the ^{13}C and ^{1}H resonances of the side chains of the aliphatic residues were obtained with C(CO)-TOCSY, H(CCO)-TOCSY and HCCH-TOCSY and the assignments of the aromatic resonances were partially completed by 2D (Hβ)Cβ(CγCδ)Hδ and (Hβ)Cβ(CγCδCε)Hε. Thus 91% of all non-labile protons have been assigned.

The CSI program by Wishart et al. (1997) was used to determine the consensus chemical shift indices of all assigned residues (Figure 1B). The data show four helices and seven β-strands. These data are fully consistent with sequential NOE data from ^{15}N NOESY-HSQC. Interstrand NOEs from ^{13}C HSQC-NOESY and preliminary structure calculations show that these strands constitute a single β-sheet. The topology of Ap_4A hydrolase is similar to the MutT enzyme (Abeygunawardana et al., 1995), but differs in several respects (two additional helices, two extra β-strands). A full analysis of the 3D solution structure of Ap_4A hydrolase is currently underway.

The ^{1}H, ^{13}C and ^{15}N assignments have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under accession number 4448.

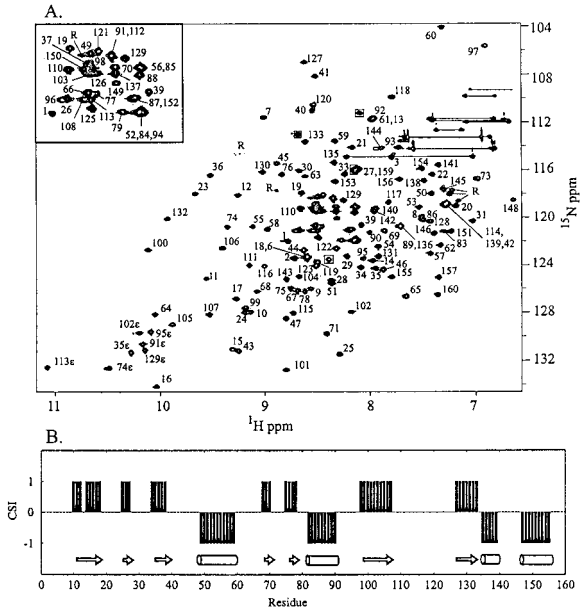

Figure 1. (A) 2D ^{1}H, ^{15}N HSQC spectrum of Ap_4A hydrolase with assigned cross peaks. The signal of Gly-109, which resonates outside of the shown region, has the chemical shifts of 5.09 ppm (^{1}H) and 108.83 ppm (^{15}N). Of the 14 Asn and Gln NH_2 side chain peaks, 10 are connected and a number of Arg side chain peaks, indicated as 'R', are folded into the spectrum. Boxed resonances are assigned to the five additional non-native residues that arise from the PreScission protease cleavage site. (B) Plot of the consensus Chemical Shift Index (CSI, ^{13}Cα, ^{13}Cβ, $^{13}C_{CO}$ and ^{1}Hα) versus residue number for Ap_4A hydrolase. Expected secondary structural elements are shown as arrows for β-strands and cylinders for helices.

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