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Letter to the Editor: ^{1}H, ^{13}C, and ^{15}N resonance assignments of the 17 kDa Ap_4A hydrolase from *Homo sapiens* in the presence and absence of ATP

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Biological context

Nudix Diadenosine 5′,5″ tetraphosphate (Ap_4A) hydrolases are ubiquitous enzymes that cleave Ap_4A asymmetrically into ATP and AMP (Guranowski, 2000). The Nudix superfamily contains over 600 members which are similar in topology and catalytic mechanism, and metabolize a wide range of nucleotide substrates (Bessman et al., 1996). On the basis of sequence similarity the asymmetric Ap_4A hydrolases can be further subdivided into 'plant' and 'animal' groups. Bacterial Ap_4A hydrolases which have been associated with pathogenic invasion of mammalian cells are most similar to the 'plant' group (Ismail et al., 2003), and those from Archea to the 'animal' group. To understand this grouping in terms of substrate binding, the NMR solution structures of Ap_4A hydrolase from the plant *Lupinus angustifolius* (Swarbrick et al., 2000; Fletcher et al., 2002) and the X-ray structures from the nematode *Caenorhabditis elegans* (Bailey et al., 2002) have been solved. These data reveal that the substrate binds in two different positions and opens avenues for the exploration of inhibitory compounds selective against the pathogenic bacterial type. However, in the *C. elegans* X-ray structure the substrate analogue has been turned over to AMP and may represent a non-native complex. Furthermore, residues highly conserved between the animal and plant groups appear to have very different roles in substrate recognition, calling into question the authenticity of substrate position in the X-ray structure. Hence, it is desirable to investigate this class distinction at the molecular level by determining the structure of a member of the 'animal' class using NMR spectroscopy. To this end, we have embarked on an investigation of the structure of the 17 kDa enzyme from *Homo sapiens* free and in complex with the natural product ATP. As a prelude to the structure we report the assignments of both the apo and ATP bound spectra.

Methods and experiments

The gene encoding residues 1–147 of human Ap_4A hydrolase (Ap_4Aase) was subcloned into the pGEX-6-P3 vector and expressed in *Escherichia coli* BL21(DE3). At millimolar concentrations wild type human Ap_4Aase precipitated within a few days preventing NMR analysis. In the course of preparing mutants of the catalytic glutamates to study their functional roles, we noted that these mutants, particularly E63A, showed improved expression, solubility and stability. As E63A gave acceptable NMR spectra, samples of this mutant were prepared for detailed NMR analysis. Isotopically labelled protein was expressed in cells grown in 1 l of minimal media supplemented with ^{13}C-glucose and ^{15}N-ammonium chloride using a 2 l fermenter at 37 °C. Cells were sonicated and debris removed by centrifugation. The supernatant was passed through a 15 ml glutathione-Sepharose 4B column and the GST fusion product was eluted and cleaved overnight with PreScission

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protease (Pharmacia) pH 8.0 and 4 °C (50 mM Tris–HCl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA). The protein was further purified on a glutathione-Sepharose and Mono-Q column eluted with a 0–250 mM NaCl gradient in 25 mM Tris–HCl at pH 8.0 and 25 °C. The final cleaved and purified protein has five additional N terminal residues (Gly, Pro, Leu, Gly, Ser) due to multiple cloning site of the pGEX-6-P3 vector and the PreScission protease recognition site.

Samples of Ap_4Aase (0.4 ml 0.7–1.0 mM) were prepared for NMR spectroscopy in buffer containing 20 mM imidazole, 20 mM $MgCl_2$, 10 mM DTT, 1 mM EDTA, 0.02% sodium azide and either 100% D_2O or 90%/10% H_2O/D_2O at pH 6.5 and sealed under nitrogen. ATP-complexed samples contained 1.5 equivalents of $^{13}C/^{15}N$ ATP. Using a Varian 600 MHz room temperature probe the following spectra were recorded at 20 °C: 2D ^{15}N fast HSQC, 3D HNCACB, CBCA(CO)NH, (H)C(CO)NH-TOCSY, H(CCO)NH-TOCSY, HNCA, HN(CO)CA, HNCO, HCCH-TOCSY (15.6 ms mixing), ^{15}N NOESY-HSQC (100 ms mixing), ^{13}C-HSQC-NOESY (110 ms mixing). Aromatic assignments were obtained from ^{13}C-NOESY-HSQC over the aromatic region and supplemented by 2D (Hβ) Cβ(CγCδ)Hδ spectra. Spectra were processed with NMRPipe (Delaglio et al., 1995) and analysed with XEASY (Bartels et al., 1995). ^1H chemical shifts were referenced to DSS at 0 ppm and ^{13}C and ^{15}N shifts were calculated from the ^1H spectrometer frequency.

Extent of assignments and data deposition

Figure 1 shows the 2D fast-^{15}N-HSQC spectrum of apo Ap_4Aase from *H. sapiens* overlayed with the spectrum with 1.5 mM ATP added. It is apparent from the absence of 11 backbone resonances in the apo spectra that the active site is undergoing significant motion. Interestingly, with the addition of ATP, these resonances appeared suggesting that the motion in the active site region is dampened by the presence of the ligand. As these changes suggest both conformational and dynamic changes have occurred on substrate binding simple chemical shift mapping does not clearly resolve the binding site of the adenine group and therefore we are determining high resolution structures of the enzyme both in the absence and presence of ATP. Most sequential backbone assignments were made from a combination of HNCA and CBCA(CO)NH spectra since the HNCACB spectra gave limited additional information. This can be attributed to a time dependent aggregation phenomenon as judged by the observation of an increase in the linewidths from older samples. Side chain assignments were established from a combination of the ^{13}C-HSQC-NOESY and HCCH-TOCSY spectra. Side chains from residues that had no amides detected in the free form were assigned from side chain spin typing and initial structures. A total of 94% (95%) and 97% (98%) of backbone and side chain assignments, respectively, in the free (and ATP bound) enzymes have been obtained and have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under Accession Numbers 6330 (and 6336).

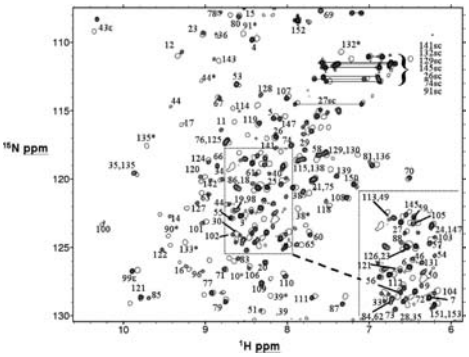

Figure 1. Overlay of 2D ^1H, ^{15}N HSQC (600 MHz) spectra of human Ap_4A hydrolase with (open contours) and without (filled) the addition of 1.5 equivalents of ATP. Assignments from the apo spectra are shown along with selected resonances from the ATP complex which are labelled with asterisks. Some of these latter resonances are broadened or not observed in the spectra of apo Ap_4Aase. Sidechain NH_2 resonances of Gln and Asn are connected.

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