Published in final edited form as:

Biomol NMR Assign. 2010 April ; 4(1): 5–8. doi:10.1007/s12104-009-9196-y.

Chemical shift assignments for human apurinic/apyrimidinic endonuclease 1

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Abstract

Apurinic/apyrimidinic endonuclease 1 (APE1 or Ref-1) is the major enzyme in mammals for processing abasic sites in DNA. These cytotoxic and mutagenic lesions arise via spontaneous rupture of the base-sugar bond or the removal of damaged bases by a DNA glycosylase. APE1 cleaves the DNA backbone 5' to an abasic site, giving a 3'-OH primer for repair synthesis, and mediates other key repair activities. The DNA repair functions are essential for embryogenesis and cell viability. APE1-deficient cells are hypersensitive to DNA-damaging agents, and APE1 is considered an attractive target for inhibitors that could potentially enhance the efficacy of some anti-cancer agents. To enable an important new method for studying the structure, dynamics, catalytic mechanism, and inhibition of APE1, we assigned the chemical shifts (backbone and $^{13}\text{C}\beta$) of APE1 residues 39-318. We also report a protocol for refolding APE1, which was essential for achieving complete exchange of backbone amide sites for the perdeuterated protein.

Keywords

APE1; Ref-1; APEX1; Apurinic/apyrimidinic endonuclease; DNA base excision repair; NMR chemical shift assignments

Biological context

Apurinic/apyrimidinic endonuclease 1 (APE1) is a multifunctional protein with essential roles in DNA repair and transcriptional regulation in mammals (Bhakat et al. 2009). APE1 is also known as Ref-1 (redox enhancing factor-1), reflecting its role in activating c-Jun, NF- κ B, and other key transcription factors, which is thought to involve the reduction of a Cys side chain in their DNA-binding domain. In addition, APE1, together with other proteins, binds to so-called negative Ca^{2+} -response elements (nCaRE) to regulate the expression of some genes, an activity that requires acetylation of APE1 (Ca^{2+} -dependent) by the transcriptional co-activator p300. APE1 initiates the repair of mutagenic and cytotoxic abasic sites in DNA, lesions that are generated by spontaneous depurination (~10,000 times per cell per day) or removal of damaged bases by a DNA glycosylase to initiate base excision repair. APE1 hydrolyses the phosphodiester bond 5' to AP sites to give a single strand break, and its 3'-phosphodiesterase activity can remove fragmented sugar moieties at the 3' end of DNA strand breaks caused by some drugs (bleomycin) or ionizing radiation. In both cases, APE1 produces a 3'-OH primer for repair synthesis by DNA polymerase pol β . APE1 is the major AP endonuclease in humans; its repair activity is essential for embryogenesis and cell viability and its suppression increases

the number of abasic sites and triggers apoptosis. APE1 confers resistance to pro-apoptotic stimuli including ionizing radiation, oxidative stress, and some chemotherapeutic agents, and suppression of APE1 sensitizes cells to DNA-damaging agents including some used to treat cancer (Fishel and Kelley 2007). Thus, inhibition of APE1 is an attractive strategy for increasing the efficacy of anti-cancer drugs, including alkylating agents and antimetabolites such as 5-fluorouracil (McNeill et al. 2009). While potent and clinically useful inhibitors of APE1 are highly desirable, none have been identified to date. Despite the significant biochemical and structural studies conducted on APE1 over the past two decades, including crystal structures of free and DNA-bound APE1 (Beernink et al. 2001; Gorman et al. 1997; Mol et al. 2000), its catalytic mechanism has not been elucidated. To enable an important new experimental approach for studying the structure, dynamics, catalytic mechanism, post-translational modification (oxidation, acetylation), and inhibition of APE1, we have assigned the chemical shifts (backbone and $^{13}\text{C}^\beta$) of perdeuterated APE1 residues 39-318.

Methods and experiments

While full length human APE1 contains 318 residues (35.6 kDa), previous structural and biochemical studies and our observations indicate that residues 1 to ~44 are disordered and are dispensable for AP endonuclease activity (Beernink et al. 2001; Gorman et al. 1997; Mol et al. 2000). Consistent with crystallographic studies showing a lack of electron density for residues 1–44, we observe ~45 ^1H - ^{15}N peaks with very strong intensity and poor dispersion in a ^{15}N -HSQC spectrum of full-length APE1 (not shown). Accordingly, we produced a plasmid for expressing residues 39-318 of APE1 (APE1 $^{\Delta\text{N}38}$) via PCR amplification from a plasmid harboring the intact human APE1 gene and sub-cloning into the *NheI* and *BamHI* sites of a pET-28 plasmid (Novagen), and verified the construct by DNA sequencing. APE1 $^{\Delta\text{N}38}$ is expressed and purified at 4°C as described for full-length APE1 (Fitzgerald and Drohat 2008), including Ni-affinity chromatography (Qiagen), overnight thrombin cleavage of the N-terminal poly-His tag (giving six non-native N-terminal residues, GSHMAS), and ion exchange using a 5 ml HiTrap SP HP column (GE Healthcare). This provides APE1 $^{\Delta\text{N}38}$ (286 residues, 32.1 kDa) that is >99% pure as judged by SDS-PAGE (commissie stained gel). APE1 $^{\Delta\text{N}38}$ is quantified by absorbance ($\epsilon^{280} = 54.4 \text{ mM}^{-1}\text{cm}^{-1}$). APE1 $^{\Delta\text{N}38}$ has the same AP endonuclease activity (k_{cat}) as full-length APE1 (not shown). Consistent with a disordered N-terminal domain, most of the very intense and poorly dispersed ^1H - ^{15}N resonances in a ^{15}N -HSQC spectrum of APE1 are not observed for APE1 $^{\Delta\text{N}38}$ (not shown).

We initially sought to assign the chemical shifts for uniformly ^{13}C , ^{15}N -labeled APE1 $^{\Delta\text{N}38}$ using the standard triple-resonance NMR experiments, but we were unable to assign more than ~40% of the backbone resonances due to insufficient signal, indicating a need for perdeuterated protein and TROSY-based NMR experiments. To produce uniformly ^2H , ^{13}C , ^{15}N -labeled APE1 $^{\Delta\text{N}38}$, we transformed BL21(DE3) cells (Novagen) with the APE1 $^{\Delta\text{N}38}$ expression plasmid, inoculated an LB plate and incubated overnight at 37°C. A single colony was added to 5 ml LB medium (H_2O) and grown at 37°C to $\text{OD}_{600} = 0.7$, then a 1.4 ml volume was centrifuged and the isolated cells added to 20 ml MOPS minimal medium (H_2O) and grown to $\text{OD}_{600} = 0.6$. Subsequently, a 4 ml volume was centrifuged and the isolated cells were added to 80 ml of MOPS- $^2\text{H}_2\text{O}$ medium prepared with 99.9% $^2\text{H}_2\text{O}$, U- ^{15}N - NH_4Cl (1 g/l), and U- ^{13}C -glucose (2 g/l) (Cambridge Isotope Laboratories). At $\text{OD}_{600} = 0.5$ the cells were diluted to 160 ml with MOPS- $^2\text{H}_2\text{O}$, grown to $\text{OD}_{600} = 0.5$, and diluted to 800 ml with MOPS- $^2\text{H}_2\text{O}$. At $\text{OD}_{600} = 0.4$ the temperature was reduced to 15°C and expression was induced with 0.4 mM isopropyl-1-thio- β -D-galactopyranoside and continued for 24 h. The ^2H , ^{13}C , ^{15}N -labeled APE1 $^{\Delta\text{N}38}$ was purified as outlined above, giving a final yield of 15 mg.

A key step in producing perdeuterated protein that is optimal for NMR studies is to replace ^2H with ^1H at backbone amide sites exhibiting slow exchange. This was important for APE1, because over 20% of the $^1\text{H}^{\text{N}}\text{-}^{15}\text{N}$ peaks were substantially diminished or absent in a 2D ^{15}N -TROSY spectrum of ^2H , ^{13}C , ^{15}N -labeled APE1 $^{\Delta\text{N}38}$ that was purified as described above, and many were still weak or absent after incubation of the protein at 30°C for several weeks (not shown). To achieve complete exchange of the backbone amide sites, we denatured APE1 $^{\Delta\text{N}38}$ (6 M Gdn-HCl) and screened for refolding conditions that would provide the highest yield of catalytically active enzyme using a previously published buffer screen (Willis et al. 2005) with some important modifications. The protocol used to prepare the NMR sample is as follows. We denatured 15 mg of pure ^2H , ^{13}C , ^{15}N -labeled APE1 $^{\Delta\text{N}38}$ in 7.7 ml of buffer containing 6 M Gdn-HCl, 0.02 M Tris-HCl pH 7.8, 0.1 M NaCl, 5 mM DTT for 1 h at room temperature with stirring. The denatured protein was rapidly diluted in 154 ml refolding buffer containing a 2.5-fold molar excess of abasic DNA (an APE1 substrate), and incubated with stirring for 1 h at room temperature. The refolding buffer included 0.05 M Tris-HCl pH 8.2, 0.55 M Gdn-HCl, 0.011 M NaCl, 0.4 mM KCl, 5 mM TCEP, 2 mM MgCl_2 , 2 mM CaCl_2 . The duplex DNA was prepared from two oligonucleotides (Keck Facility, Yale University), 5'-TCAFGTACAGA GCTGC (*F* is the tetrahydrofuran abasic site analog) and 5'-AGTGCATGTCTCGACG, which were purified and quantified as described (Fitzgerald and Drohat 2008). The refolded protein was dialyzed 16 h at 4°C versus ion exchange buffer (25 mM HEPES pH 7.5, 0.15 M NaCl, 1 mM DTT, 0.2 mM EDTA, 1% glycerol), and the DNA was fully removed by ion exchange chromatography, conducted twice, using a 5 ml HiTrap Q Sepharose HP column (GE Healthcare). The purified APE1 $^{\Delta\text{N}38}$ was dialyzed against NMR buffer (0.02 M sodium phosphate pH 6.5, 0.1 M NaCl, 0.5 mM DTT, 0.2 mM EDTA) overnight at 4°C and then concentrated. The complete removal of DNA and proper refolding was demonstrated by our observation that the A_{280}/A_{260} ratio and ^{15}N -TROSY spectrum for refolded APE1 $^{\Delta\text{N}38}$ is identical to that observed for APE1 $^{\Delta\text{N}38}$ that had not been denatured or exposed to DNA. Moreover, the AP endonuclease activity (k_{cat}) is the same for APE1 $^{\Delta\text{N}38}$ that had and had not been denatured and refolded (not shown).

The NMR sample (0.33 ml) consisted of 0.8 mM APE1 $^{\Delta\text{N}38}$ in NMR buffer with 10% $^2\text{H}_2\text{O}$. The NMR experiments were performed at 298 K on a Bruker AVANCE 800 MHz NMR spectrometer equipped with a 5 mm triple-resonance cryogenic probe with *z*-axis pulse field gradients. We collected a 2D ^{15}N -edited TROSY and TROSY-based versions of the standard triple resonance experiments, including the HNCA, HN(CO)CA, HNCACB, HN(CO)CACB, HNCO, and HN(CA)CO (Salzmann et al. 1999). NMR data were processed with NMRPipe (Delaglio et al. 1995) and analyzed with Sparky (Goddard and Kneller). The MARS program was used to obtain initial chemical shift assignments for many residues (Jung and Zweckstetter 2004). The ^1H chemical shifts were referenced to external DSS, the ^{13}C shifts were referenced indirectly to DSS using the frequency ratio $^{13}\text{C}/^1\text{H} = 0.251449530$, and the ^{15}N shifts were referenced indirectly to liquid ammonia using $^{15}\text{N}/^1\text{H} = 0.101329118$.

Extent of assignments and data deposition

Analysis of the triple resonance experiments provided the sequence-specific assignments for over 90% of the backbone ($^1\text{H}^{\text{N}}$, ^{15}N , $^{13}\text{C}^{\alpha}$, $^{13}\text{C}'$) and $^{13}\text{C}^{\beta}$ resonances for APE1 $^{\Delta\text{N}38}$. Figure 1 shows a ^{15}N -TROSY spectrum of APE1 $^{\Delta\text{N}38}$ with the backbone ^1H - ^{15}N resonances assigned. We assigned 235 of 261 (90%) of the backbone $^1\text{H}^{\text{N}}$ - ^{15}N resonances (APE1 $^{\Delta\text{N}38}$ has 280 native residues with 19 Pro), and 94% of $^{13}\text{C}^{\alpha}$, 93% of $^{13}\text{C}^{\beta}$, and 93% of $^{13}\text{C}'$ resonances. We assigned the $^{13}\text{C}^{\alpha}$, $^{13}\text{C}^{\beta}$, and $^{13}\text{C}'$ resonances for all Pro residues except Pro48, which is followed by Pro49, and Pro122 located in a loop region. The backbone $^1\text{H}^{\text{N}}$ - ^{15}N chemical shifts could not be assigned for residues G80-V84, E87, D90, E101, L104, S120, S123-G130, Q153-R156, G178, D210, T268, and G279. These residues are located in loops or regions for which low electron density or high temperature (*B*) factors are observed in one or more of the three crystal

structures of free APE1 (Beernink et al. 2001; Gorman et al. 1997). The absence of backbone $^1\text{H}^{\text{N}}$ - ^{15}N resonances for residues in helix 1 (80-87) is consistent with evidence of mobility from crystallographic studies (higher than average B factors) and molecular dynamics simulations (Beernink et al. 2001). It was proposed that mobility of this helix may expose the buried Cys65 side chain, which has been proposed to play a role in the redox activation of transcription factors by APE1 (Beernink et al. 2001). As shown in Fig. 2, the secondary structure predicted by the program TALOS+ (Shen et al. 2009) using our assigned chemical shifts is in close agreement with crystal structures of APE1. Our assignment of the $^{13}\text{C}^{\alpha}$ and $^{13}\text{C}^{\beta}$ chemical shifts for all seven Cys residues of APE1 will facilitate NMR studies into the potential role of Cys oxidation in regulating its DNA repair activity and in its redox-mediated activation of transcription factors including c-Jun and NF- κ B. We have also assigned chemical shifts for three residues essential for AP endonuclease activity, Tyr171, D210 (except $^1\text{H}^{\text{N}}$, ^{15}N) and His309, which could facilitate future NMR studies of their role in catalysis. The chemical shift assignments for APE1 $^{\Delta\text{N}38}$ have been deposited in the BioMagResBank under the accession number 16516.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (R01-GM72711 to A.C.D.) and the University of Maryland Marlene and Stewart Greenebaum Cancer Center. B.M. is supported by a Chemistry-Biology Interface (CBI) Training grant from the NIH, T32-GM066706. The NMR spectrometers used in these studies were purchased, in part, with funds from shared instrumentation grants from the NIH (S10-RR10441; S10-RR15741; S10-RR16812; S10-RR23447) and the NSF (DBI 0115795). We thank Mike Morgan for producing the expression plasmid for APE1 $^{\Delta\text{N}38}$.

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Fig. 1. Shown is the 2D ^{15}N -TROSY spectrum of ^2H , ^{13}C , ^{15}N -labeled APE1 $^{\Delta\text{N}38}$ (0.8 mM) collected at 298 K on an 800 MHz NMR spectrometer. For clarity, the region shown does not include G231 ($\delta^1\text{H} = 6.23$, $\delta^{15}\text{N} = 118.3$)

Fig. 2.

Secondary structure of APE1 predicted by TALOS+ using the chemical shifts reported here agrees with that observed in crystal structures of APE1. The output from the program TALOS+ (Shen et al. 2009) is displayed as a column chart (*blue*, β -strand; *red*, α -helix) and column height reflects the probability assigned by the prediction program of TALOS+ (α -helical values are negative for clarity). Shown above the column chart is the secondary structure observed in APE1 crystal structures (PDB ID: 1BIX, 1HD7)