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# The role of Asn-212 in the catalytic mechanism of human endonuclease APE1: Stopped-flow kinetic study of incision activity on a natural AP site and a tetrahydrofuran analogue

Lyubov Yu. Kanazhevskaya[a,b], Vladimir V. Koval[a,b], Alexander A. Lomzov[a,b], Olga S. Fedorova[a,b,*]

[a] Institute of Chemical Biology & Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, Lavrentyev Ave., 8, Novosibirsk 630090, Russia
[b] Novosibirsk State University, Pirogova St., 2, Novosibirsk 630090, Russia

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ABSTRACT

Mammalian AP endonuclease 1 is a pivotal enzyme of the base excision repair pathway acting on apurinic/apyrimidinic sites. Previous structural and biochemical studies showed that the conserved Asn-212 residue is important for the enzymatic activity of APE1. Here, we report a comprehensive pre-steady-state kinetic analysis of two APE1 mutants, each containing amino acid substitutions at position 212, to ascertain the role of Asn-212 in individual steps of the APE1 catalytic mechanism. We applied the stopped-flow technique for detection of conformational transitions in the mutant proteins and DNA substrates during the catalytic cycle, using fluorophores that are sensitive to the micro-environment. Our data indicate that Asn-212 substitution by Asp reduces the rate of the incision step by ~550-fold, while Ala substitution results in ~70,000-fold decrease. Analysis of the binding steps revealed that both mutants continued to rapidly and efficiently bind to abasic DNA containing the natural AP site or its tetrahydrofuran analogue (F). Moreover, transient kinetic analysis showed that N212A APE1 possessed a higher binding rate and a higher affinity for specific substrates compared to N212D APE1. Molecular dynamics (MD) simulation revealed a significant dislocation of the key catalytic residues of both mutant proteins relative to wild-type APE1. The analysis of the model structure of N212D APE1 provides evidence for alternate hydrogen bonding between Asn-212 and Asp-210 residues, whereas N212A possesses an extended active site pocket due to Asn removal. Taken together, these biochemical and MD simulation results indicate that Asn-212 is essential for abasic DNA incision, but is not crucial for effective recognition/binding.



## 1. Introduction

The human AP endonuclease 1 (APE1) exhibits an endonuclease activity towards apurinic/apyrimidinic (AP) sites during base excision repair (BER). The AP site produced by DNA glycosylase activity at the first step of the BER pathway is subsequently recognized by APE1, which incises the phosphodiester backbone of DNA immediately 5′ to the lesion. The resulting 3′-OH moiety is processed by DNA polymerase β [1,2]. In addition to endonucleolytic activity, APE1 exhibits 3′–5′ exonuclease and 3′-phosphodiesterase activities [3], which play a significant role in 3′-phosphate and 3′-phosphoglycolate removal, also called "blocking" termini [4]. The structure and enzymatic mechanism of APE1 posed major research areas over the past twenty years. The Protein Data Bank [5] contains eight different crystal structures of the APE1 protein on its own, and complexed with either the substrate or product. According to the structure reported by Tainer et al. [6], APE1 inserts loops into both the major and minor DNA grooves, and binds to a flipped-out AP site in a pocket bordered by hydrophobic and aromatic amino acids. It is important that APE1 mainly contacts the damaged strand of the dsDNA. The APE1-bound DNA is substantially distorted in relation to the B-DNA helix. Stabilization of the kinked abasic substrate is mediated by interactions of one of the APE1 α-helixes with the minor groove, and by the insertion of Met-270, Met-271 and Arg-177 through the minor and major

*Abbreviations:* APE1, AP endonuclease1; WT, wild type; BER, base excision repair; AP, abasic site; F, 2-hydroxymethyl-3-hydroxy-tetrahydrofuran; 2-aPu, 2-aminopurine; ODN, oligodeoxyribonucleotide; MD, molecular dynamics.

* Corresponding author at: Institute of Chemical Biology & Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, Lavrentyev Ave., 8, Novosibirsk 630090, Russia. Tel.: +7 383 363 51 75; fax: +7 383 363 51 53.
*E-mail address:* fedorova@niboch.nsc.ru (O.S. Fedorova).

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grooves to occupy the place of the flipped-out AP site. An extensive site-directed mutagenesis study of APE1 showed that the side chains of Asn-226, Asn-229 and Arg-177, interacting with two downstream AP-DNA phosphates, provide specific binding to abasic DNA [7]. Finally, the abasic sugar moiety is stabilized within the hydrophobic active site pocket, which includes the side chains of Phe-266, Trp-280 and Leu-282. Formation of the enzyme's catalytically active state principally depends on the presence and location of the $Mg^{2+}$ ion. This is coordinated by oxygen atoms of the 5′-phosphate moiety as well as by Glu-96 and Asp-70 via a water molecule in the first hydration shell of the metal ion. Previous crystallographic and computational studies suggested two alternative reaction mechanisms: one predicts the presence of two metal ions in the APE1 active site for catalysis [8], while the other model suggests that $Mg^{2+}$ moves from the B-site (Asp-210, Asn-212 and His-309) to the A-site (Asp-70 and Glu-96) during the catalytic cycle [9]. However, the recently published high resolution crystal structure of free APE1 with the essential $Mg^{2+}$ cofactor has confirmed the presence of a single metal ion in the A-site, while the B-site is most probably occupied by a water molecule [10]. The authors also suggest that the water molecule, coordinated by Asn-212 and Asp-210 side chains, serves as a nucleophile for phosphodiester bond hydrolysis. This idea is supported by recently published work, which reported the crystal structure of an APE1-product complex [11]. Additionally, this study provides molecular dynamics simulations of wild type APE1 bound to abasic DNA and its important mutants E96A, Y171P, H309A, N212A, D210N and H309N. These findings show that the phosphate intermediate is stabilized by the $Mg^{2+}$ ion, and contacts His-309 and Tyr-171. Meanwhile, the side chains of Asn-212 and Asp-210 coordinate a water molecule, generating a nucleophile for attacking the phosphodiester bond. Several biochemical studies have described the significance of the conserved Asn-212 residue for APE1 catalytic activity. Rothwell et al. [12] published the first paper on Asn-212 mutants, which reported the important function of Asn-212 in AP site recognition/binding, and incision by APE1 enzyme. Further study demonstrated that substitution of Asn-212 with alanine resulted in a 7000-fold decrease in the processing rate of 52-nt F-containing DNA duplexes [11]. Besides its contribution to catalysis, the Asn-212 residue mediates interactions between APE1 and human DNA glycosylase MYH [13]. A recent bioinformatics study identified possible variations in the APE1 gene that may, in some cases, affect the stability and folding of the enzyme [14]. Particularly, N212H and N212K mutations were found to be deleterious to the function of the protein, indicating the crucial role of the Asn-212 residue.

In our previous studies [15–17], the stopped-flow pre-steady-state kinetic analysis, combined with measurements of the protein tryptophan and DNA 2-aminopurine fluorescence, was successfully applied to detect and quantitatively describe conformational transitions of the enzyme–DNA complex corresponding to particular steps of APE1 reaction. As a result, a four-step kinetic mechanism was proposed, which includes two-step binding, an irreversible substrate cleavage step and dissociation of the enzyme–product complex. Here, the same approach was utilized to investigate the role of the Asn-212 residue in specific steps of APE1 catalysis. A detailed transient kinetic analysis of N212D and N212A APE1 mutants was performed to measure the effect of Asn-212 substitutions on the rates of binding and incision of the specific substrates. Our data showed a substantial reduction of the AP-DNA incision rate by N212D APE1, and practically no incision activity for the N212A mutant. Importantly, the N212A mutant possesses a higher binding affinity to specific substrates compared to the mutant with Asn→Asp substitution.

To obtain a greater understanding of the pre-steady-state kinetic data, we performed MD simulations of WT and mutant APE1 in complex with AP- or F-containing DNA substrates. The findings demonstrated a large distortion of the active site geometry of N212D and N212A mutants, resulting from the dislocation of the key amino acid residues. We found that substitution of Asn-212 with Ala leads to the disruption of the hydrogen bonding network between Asn-212, Asp-210, and catalytic water, whereas the presence of carboxylate at position 212 maintains this network, albeit strongly modified. Thus, this combined biochemical and computational study has elucidated Asn-212's role, during the steps of recognition/binding and incision of abasic DNA by APE1, in more detail.

**Table 1**
Oligodeoxyribonucleotide substrates utilized in this study.

| Substrate | Sequence[a] |
|---|---|
| AP | 5′ CTCTCAPCCTTCC 3′<br>3′ GAGAGCGGAAGG 5′ |
| F | 5′ CTCTCFCCTTCC 3′<br>3′ GAGAGCGGAAGG 5′ |
| AP(2-aPu) | 5′ CTCTCAP(2-aPu)CTTCC 3′<br>3′ GAGAGCCGAAGG 5′ |
| F(2-aPu) | 5′ CTCTCF(2-aPu)CTTCC 3′<br>3′ GAGAGCCGAAGG 5′ |

[a] AP, natural abasic site; F, 2-hydroxymethyl-3-hydroxy-tetrahydrofuran; 2-aPu, 2-aminopurine.

## 2. Materials and methods

### 2.1. Oligonucleotides

Oligodeoxyribonucleotides (ODNs; Table 1) containing normal DNA bases, deoxyuridine, tetrahydrofuran, and 2-aminopurine were synthesized on an ASM-700 synthesizer (BIOSSET Ltd., Novosibirsk, Russia) using phosphoramidites, purchased from Glen Research (Sterling, VA) and purified by the anion exchange high-performance liquid chromatography (HPLC) on a Nucleosil 100-10 $N(CH_3)_2$ column followed by the reverse-phase HPLC on a Nucleosil 100-10 $C_{18}$ column (both columns from Macherey-Nagel, Düren, Germany). Concentrations of the ODNs were determined from their absorbance at 260 nm. The AP-containing ODN was prepared by incubation of the deoxyuridine-containing ODN (0.1 mmol) for 14 h at 37 °C with 15 U of uracil-DNA glycosylase in 150 μl of 20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin, as previously described. The reverse phase HPLC on a Nucleosil 100-5 $C_{18}$ column was used to purify the reaction product, which had a shorter retention time than the starting oligonucleotide, using a linear gradient of 0–20% acetonitrile in 0.1 M triethylammonium acetate (pH 7.0). The pooled fractions were concentrated and then converted to the lithium salt form using a Sep-Pak Plus $C_{18}$ cartridge (Waters, Milford, MA, USA). To confirm the presence of the AP site in the ODN after the treatment with uracil-DNA glycosylase, samples were mixed with 10% aqueous piperidine at 95 °C, or annealed to the complementary oligonucleotide and treated with APE1 under the conditions described below. A PAGE analysis indicated that in both cases the material was cleaved to two shorter oligonucleotides. The purity, homogeneity, and integrity of each ODN were proved by MALDI-TOF mass spectroscopy and assessed by 20% PAGE after staining with Stains-All (Sigma–Aldrich, St. Louis, MO). When needed, the modified DNA strands were $^{32}P$-labelled using [γ-$^{32}P$]-ATP and bacteriophage T4 polynucleotide kinase (SibEnzyme, Russia) according to the manufacturer's protocol, and purified by 20% denaturing PAGE. ODN duplexes were prepared by annealing the modified and complementary strands in a molar ratio of 1:1. Under the conditions of stopped-flow experiments with a high $Mg^{2+}$ concentration (see below), the predicted equilibrium constant of the duplex formation is about $1 \times 10^9\ M^{-1}$, suggesting that nearly 100% of the oligonucleotides are in the double helix form [18–20].