

## Comparative analysis of exo- and endonuclease activities of APE1-like enzymes

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**Keywords:** DNA repair, apurinic/apyrimidinic endonuclease, abasic site, damaged nucleotide, endonuclease activity, 3'-5' exonuclease activity.

### Abstract

Apurinic/apyrimidinic (AP) endonucleases are multifunctional enzymes that are required for cell viability. AP endonucleases cleave DNA 5' to an AP site; can recognize and process some damaged nucleosides; and also possess 3'-phosphodiesterase, 3'-phosphatase, and endoribonuclease activities. To deeply insight the mechanism of substrate cleavage we have analyzed the effect of mono- and bivalent metal ions on the exo- and endonuclease activities of four homologous APE1-like endonucleases from insect, amphibian, fish and human. It was found that enzymes have similar dependence on metal ions concentration in the case of AP endonuclease activity supporting that the main biological function on AP site cleavage is highly conserved for evolutionary distant species. Obtained data demonstrate that the efficiency of 3'-5' exonuclease activity was significantly higher for hAPE1. Otherwise, the endoribonuclease activity of enzymes increases in the order hAPE1 ~ zAPE1 ≤ xAPE1 ≤ Rrp1. Taken together, obtained data revealed that tested enzymes demonstrate significant variety in the ability of substrate cleavage despite the most important catalytic and substrate binding amino acid residues are identical. It could be concluded that substrate specificity and cleavage efficiency is controlled by external to catalytic site factors, such as N-terminal domain of these enzymes.

## Introduction

Apurinic/aprimidinic (AP) endonucleases are essential enzymes for abasic site cleavage in the BER pathway [1–3]. As the result of AP-endonuclease action, a single-nucleotide gap with 3'-OH and 5'-phosphate is formed [4,5]. However, AP-endonucleases can recognize not only AP-sites, but also some damaged nucleotides containing a modified base, and catalyze hydrolysis of the phosphodiester bond on the 5'-side of the damaged nucleotide. The activity of AP-endonucleases toward a damaged-base-containing DNA was initially reported for Nfo from *E. coli* [6–9] and later described for human APE1 [10–12] and other APE1-like enzymes [13]. AP-endonucleases can recognize various damaged nucleotides such as 5,6-dihydrouridine (DHU),  $\alpha$ -anomers of nucleotides, 1, $N^6$ -ethenoadenosine ( $\epsilon$ A), uracil (U), and other modified residues [10,14]. Moreover, they have 3'-5' exonuclease [15–17] and endoribonuclease [18–21] activities toward non-damaged DNA and RNA, respectively.

The successful characterization of crystal structures of human APE1 [22–25] bound with DNA revealed features of enzyme-DNA contacts. Such the DNA-binding site consists of Arg73, Ala74, Lys78, Tyr128, Arg156, Arg181, Asn222, Asn226, and Thr268. Human APE1 possesses two amino acid residues, Arg177 and Met270, which are inserted into the DNA helix after eversion of damaged nucleotide. Amino acid residues Asp70, Glu96, Tyr171, Asp210, Asn212, Asp308, and His309 are responsible for the coordination of the cofactor-ion  $Mg^{2+}$  and scissoring of phosphate group of the damaged nucleotide. Despite the successful characterization of crystal structures of human APE1 bound with various metal ions as well as substrate/product the location, the stoichiometry and the catalytic function of the divalent metal cation are still being debated [22,26,35,27–34]. Such, the metal-binding site identified in the first reported APE1 crystal structure containing one  $Sm^{3+}$  ion in the active site, which is often referred to as the “A-site” [26]. Two structures of DNA-free APE1 were reported with  $Pb^{2+}$  ions in the active site [27]. Both of these structures have a  $Pb^{2+}$  ion at the A-site, and one of the structures has the second  $Pb^{2+}$  ion, at the “B-site,” coordinated by residues Asp-210, Asn-212, and His-309. A “moving-metal mechanism” was proposed where one  $Mg^{2+}$  ion moves from the “B-site” to the “A-site” during substrate cleavage [30]. In later studies, the structure of human APE1 was solved at 1.92 Å resolution with a single  $Mg^{2+}$  ion in the active site [28]. The structure reveals ideal octahedral coordination of  $Mg^{2+}$  via two carboxylate groups (Asp-70 and Glu-96) and four water molecules. The binding site for  $Mg^{2+}$  in the new structure is the same as that observed for the surrogate

metals  $\text{Sm}^{3+}$  and  $\text{Pb}^{2+}$  in previously published structures of DNA-free APE1. The latest structural studies on human APE1 showed that repositioning of  $\text{Mg}^{2+}$  is facilitated by the structural plasticity of Glu-96 in the active site of the enzyme [33]. Structures of an enzyme–product (EP) complex and enzyme–substrate (ES) complex with a single  $\text{Mg}^{2+}$  ion in the active site were determined recently [25,36]. In the EP complex,  $\text{Mg}^{2+}$  is coordinated directly by Glu-96, the 3'-OH, a non-bridging O atom of the nascent 5'-phosphate, and by three water molecules, one of which is bound to Asp-70.

The role of the metal ion and several catalytic mechanisms were recently carefully examined by the molecular mechanics methodology [37]. It was suggested that  $\text{Mg}^{2+}$ -bound water triggers leaving group departure by neutralizing the 3'-hydroxyl of the neighboring nucleotide. It was suggested that metal ion facilitates the departure of the leaving group through proton transfer from a water molecule. His-309, as well as Tyr-171 and Asn-212, stabilize the increased charge forming on the substrate backbone as the reaction proceeds. Asn-212 and Tyr-171 also play key roles at the start of the reaction by positioning the Asp-210 base and the nucleophilic water molecule in the active site, respectively. Although it was previously suggested [38,39] that His-309 should be neutral and initiate the reaction by activating a water nucleophile, calculations [37] demonstrate that His-309 must be protonated to help neutralize the charge on the phosphorane intermediate, which is in good agreement with NMR and crystallographic data [25,36,40].

Recently, the mechanism of target nucleotide recognition by human AP-endonuclease APE1 was suggested based on the pulsed electron–electron double resonance (PELDOR) spectroscopy [41] and pre-steady-state kinetic analysis of enzyme and DNA conformational changes during DNA binding [35,42,43]. The plasticity of the active site pocket, which allow to accommodate different damaged nucleotides, was found by MD simulations [43]. Summarizing the common strategy of target nucleotide recognition mechanism of AP-endonucleases was hypothesized [41,44]. In this mechanism the non-specific contacts between amino acid residues of the DNA binding site and DNA backbone serve to sculpt DNA structure for bending the double helix, local DNA melting and damaged-nucleotide eversion.

Despite of the common strategy of target nucleotide recognition recently was found [13] that high homologues APE1-like enzymes have a different activity toward DNA containing damaged bases. It was revealed significant variety of damaged DNA cleavage efficacy by

comparative kinetic analysis of homologous APE1-like endonucleases human APE1, insect *Drosophila melanogaster* Rrp1, amphibian *Xenopus laevis* xAPE1, and fish *Danio rerio* zAPE1 during their interaction with DNA-substrates containing an F-site (an analog of an AP-site), 1,*N*<sup>6</sup>-ethenoadenosine ( $\epsilon$ A), 5,6-dihydrouridine (DHU), uridine (U), or the  $\alpha$ -anomer of adenosine ( $\alpha$ A). An alignment of sequences of the catalytic domain of the four AP endonucleases revealed that almost all DNA-binding amino acid residues are identical among all these enzymes, except Arg181 (amino acid numbering corresponding to hAPE1 sequence), which is replaced by Asn in Rrp1, and Asn229 is replaced by Thr in xAPE1. Intercalating (Arg177 and Met270) and catalytic residues were also identical, with a single substitution of Asp70, which coordinates a  $Mg^{2+}$  ion, by Ala in the case of Rrp1. It is worth noting that two amino acid residues of the damaged-base-binding pocket (Asn229 and Ala230) are replaced by Thr and Pro in xAPE1. Finally, it was suggested that the key factor of damage recognition and cleavage efficacy associated with the fine conformational tuning inside the active site. Therefore, the conformational rearrangements inside the active site must be the driving force behind the processes catalyzed by APE1-like enzymes.

To deeply insight the mechanism of target nucleotide recognition and cleavage by homologous enzymes in the present study we have analyzed the effect of mono- and bivalent metal ions on the exo- and endonuclease activity of four APE1-like endonucleases from human APE1, insect *Drosophila melanogaster* Rrp1, amphibian *Xenopus laevis* xAPE1, and fish *Danio rerio* zAPE1. Direct detection of the product formation by polyacrylamide gel electrophoresis (PAGE) allowed to estimate the efficiency of enzymatic hydrolysis of model substrates. The model DNA-substrate containing an abasic site as well as non-damaged DNA-duplex containing 5'-dangling end and short hairpin-folded RNA-substrate were used to compare AP-endonuclease, 3'-5' exonuclease and endoribonuclease activities of four AP-endonucleases at various concentrations of metal ions.

## **Materials and Methods**

### ***Cloning of AP-endonucleases***

Synthetic codon optimized genes of the zAPE1 from *Danio rerio*, xAPE1 from *Xenopus laevis* and Rrp1 from *Drosophila melanogaster* were used to construct the expression plasmids. Genes of these enzymes were digested with BamHI and NdeI/NheI restriction sites and separated on 1.0% agarose/TAE gels, excised and purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). The gene was cloned into the pET28c expression vector digested by the same restriction enzymes. Ligation products were electroporated into ElectroMAX™ DH10B Competent Cells (Invitrogen, Waltham, Massachusetts, USA), purified by Plasmid Miniprep Kit (Merck, Darmstadt, Germany) and sequenced. The obtained constructs express zAPE1, xAPE1 and Rrp1 proteins with the N-terminal His-tag.

### ***Enzymes purification***

Human AP-endonuclease APE1 was expressed and purified in its native form without tags or other modifications as described previously [42,45]. For expression of the recombinant zAPE1, xAPE1 and Rrp1 enzymes, 1 L of *Escherichia coli* strain Rosetta II(DE3) culture (Invitrogen, France) (in Luria–Bertani [LB] broth) carrying the pET28c-APE1 construct was grown at 50 µg/mL kanamycin and 37°C until absorbance at 600 nm (A600) reached 0.6–0.7. Enzyme expression was induced overnight at room temperature with 0.3 mM isopropyl-β-D-thiogalactopyranoside. The cells were then harvested by centrifugation and suspended in 30 ml of buffer I (20 mM HEPES–KOH pH 7.8, 40 mM NaCl, 0.1% NP40) containing a protease inhibitor cocktail (Complete, Germany). The cells were lysed using a Thermo French Pressure Cell Press. The homogenate was centrifuged at 40,000 × g for 45 min. Supernatant has been brought to 250 mM NaCl (400 mM in case of Rrp1) and passed through a column packed with 25 ml of Q-Sepharose Fast Flow (Cytiva, GE Healthcare Life Sciences, USA) pre-equilibrated in the same buffer. The flow-through fractions containing the enzyme were pooled and loaded onto a 1 ml HiTrap Chelating HP™ column (Cytiva GE Healthcare Life Sciences, USA) charged with Ni<sup>2+</sup> in buffer II (20 mM HEPES-NaOH, pH 7.8, 500 mM NaCl and 20 mM imidazole). Bound proteins were eluted in a linear gradient of 20–500 mM imidazole. All purification procedures were carried out at 4°C. The protein concentration was measured by the Bradford method; the stock solution was stored at -20°C in 50% glycerol.

### ***Oligodeoxynucleotides (ODNs)***

The synthesis of the oligonucleotides (**Table 1**) was carried out on an ASM-800 DNA/RNA synthesiser (Biosset, Russia) using standard commercial phosphoramidites and CPG solid supports from Glen Research (USA). The oligonucleotides were deprotected according to manufacturer protocols and purified by HPLC. Oligonucleotide homogeneity was checked by 20% denaturing polyacrylamide gel electrophoresis (PAGE). Concentrations of oligonucleotides were calculated from their  $A_{260}$ . ODN duplexes were prepared by annealing oligonucleotide strands at a 1:1 molar ratio.

**Table 1.** DNA substrates used in this study.<sup>a</sup>

F-substrate	5'-FAM-GCTCAFGTACAGAGCTG-3' 3'-CGAGTGCATGTCTCGAC-BHQ1-5'
Exo-substrate	5'-FAM-CAGCTCTGTACGTGAGC-3' 3'-GTCGAGACATGCACTCGTCACCACTGTG-5'
RNA-substrate	5'-FAM-r(AUAUAACAUCAUUAUUAU)-BHQ1-3'

<sup>a</sup> FAM is 6-carboxyfluorescein, BHQ1 is black hole quencher, F is (2*R*,3*S*)-2-(hydroxymethyl)-3-hydroxytetrahydrofuran.

### ***Polyacrylamide gel electrophoresis (PAGE) experiments***

Single-turnover enzyme assays were performed in the different reaction buffers consist of 50 mM Tris-HCl, pH 7.5, 0-300 mM KCl, 0.0-10.0 mM MgCl<sub>2</sub>, 1.0 mM dithiothreitol, 0.0-1.0 mM EDTA and 7% glycerol (v/v). AP-endonuclease assays with all substrates were carried out at 25°C in 10 µl reaction mixture. Because AP-endonuclease and endoribonuclease activities have different characteristic time of the substrate cleavage, the reaction mixtures were incubated different time and the reaction was conducted at different concentrations of reactants (**Table 2**). The reaction was initiated by the addition of the enzyme. The reaction was quenched with 10 µl of gel-loading dye containing 7 M urea and 25 mM EDTA followed by the heating to 95°C for 3 min and loaded on a 20% (w/v) polyacrylamide/7 M urea gel. The hydrolysis of the model RNA-substrates by RNase A was performed to visualize cleavage products at pyrimidine-purine sequence context. RNase A reaction mixture (20 µl) composed of a 3.0 µM substrate and 3.0 nM RNase A in a buffer [50 mM Tris-HCl (pH 8.5), 50 mM NaCl, 1.0 mM EDTA, 1.0 mM DTT, and 9% of glycerol] was incubated at 25°C for 5 min,

quenched by 20  $\mu\text{l}$  of a gel loading solution (7 M urea and 25 mM EDTA) and incubated at 96°C for 5 min to stop the reaction.

PAGE was performed under denaturing conditions (7 M urea) at 55°C and a voltage of 200–300 V. The gels were visualized using an E-Box CX.5 TS gel-documenting system (Vilber Lourman, France) and quantified in the Gel-Pro Analyzer software (Media Cybernetics, Rockville, MD).

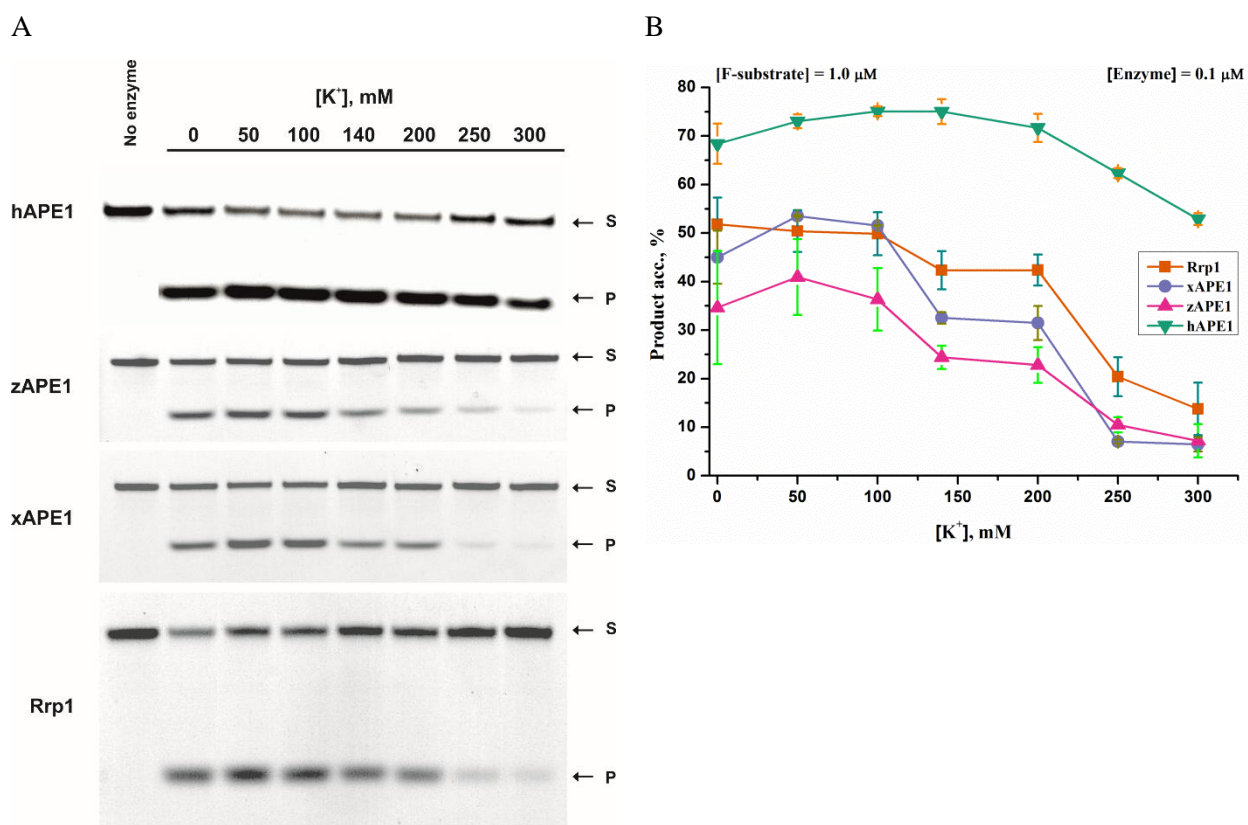
**Table 2.** Reaction conditions for comparison cleavage activity of APE1-like enzymes.

Substrate	Reactant concentrations	Reaction conditions
F-substrate	[enzyme] = 1.0 $\mu\text{M}$ , [substrate] = 1.0 $\mu\text{M}$	1) $[\text{MgCl}_2]$ = 5.0 mM, $[\text{KCl}]$ = 0 – 300 mM, 25°C, 20 s 2) $[\text{MgCl}_2]$ = 0 – 10 mM, $[\text{KCl}]$ = 50 mM, 25°C, 20 s
Exo-substrate	[enzyme] = 1.0 $\mu\text{M}$ , [substrate] = 1.0 $\mu\text{M}$	1) $[\text{MgCl}_2]$ = 5.0 mM, $[\text{KCl}]$ = 50 mM, 25°C, 30 min 2) $[\text{MgCl}_2]$ = 5.0 mM, $[\text{KCl}]$ = 0 – 300 mM, 25°C, 30 min 3) $[\text{MgCl}_2]$ = 0 – 10 mM, $[\text{KCl}]$ = 50 mM, 25°C, 30 min
RNA-substrate	[enzyme] = 2.0 $\mu\text{M}$ , [substrate] = 1.0 $\mu\text{M}$	$[\text{MgCl}_2]$ = 0.0 or 5.0 mM, $[\text{KCl}]$ = 50 mM, 25°C, 1 h

### 3. Results

#### 3.1 AP-endonuclease activity

To ascertain the nonspecific electrostatic effect of monovalent cations ( $K^+$ ) on the cleavage efficacy of F-site-containing DNA duplex by APE1-like enzymes PAGE analysis of product accumulation was carried out (Fig. 1). Obtained data revealed that AP-endonuclease activity of all tested enzymes has wide bell-shaped dependence on the concentration of  $K^+$  ions, which is fully agree with previously reported pre-steady state study of DNA binding and catalysis of hAPE1 [34].

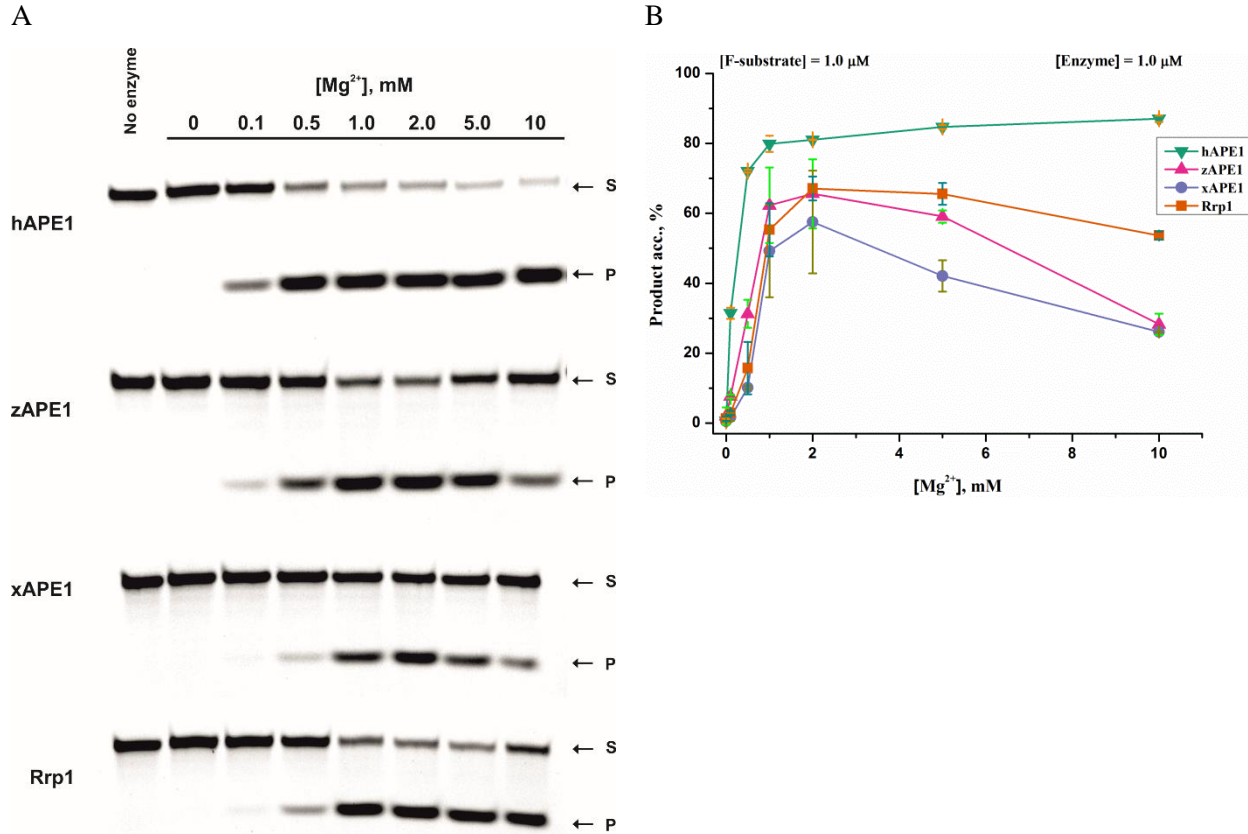


**Figure 1.** Influence of monovalent cations ( $K^+$ ) on the AP-endonuclease activity of enzymes. Accumulation of the reaction product P from F-substrate S as determined by PAGE (A). Dependences of the cleavage efficacy on  $K^+$  concentration (B). Errors were calculated by average each data point over at least three replicates.



In the case of zAPE1, xAPE1 and Rrp1 enzymes their activity was significantly weaker, than activity of hAPE1, the most efficient cleavage occurred in the presence 50–100 mM of  $K^+$ . Activity of xAPE1 and Rrp1 was approximately the same and substrate conversion reached ~50%. At the same  $K^+$  range in the case of zAPE1 only ~40% of substrate S was cleaved to form the product P (Fig. 1B). With a further increase in concentration of  $K^+$  ions up to 200 mM, the activity of enzymes towards the F-containing substrate was not changed noticeably for hAPE1 and visibly decreased for other three enzymes corresponding to order: Rrp1>xAPE1>zAPE1. With the increase of  $K^+$  concentration till 300 mM the activities of these three enzymes were noticeably decreased. The enzyme hAPE1 exhibited the same level of the F-site cleavage in the presence 50–200 mM of  $K^+$ , and the activity was slightly reduced with further increasing of the  $K^+$  concentration.

The effect of  $Mg^{2+}$  ion concentration on the F-site containing DNA cleavage was analyzed by PAGE (Fig. 2). In the case of hAPE1, the efficacy of product accumulation increases up to 1.0 mM concentration and was stable within 1.0-10 mM range. Otherwise, other APE1-like enzymes demonstrate bell-shaped dependences with maximum at 1.0-2.0 mM  $MgCl_2$ . With the further increase of  $Mg^{2+}$  concentration the activity of Rrp1 was decreased slowly than that one of xAPE1 and zAPE1 resulting to ~60% of substrate conversion to the product in the case of Rrp1 and ~30% for xAPE1 and zAPE1 at 10 mM  $Mg^{2+}$ . The inhibition of DNA cleavage by high  $Mg^{2+}$  concentration could be associated with the metal binding at divers binding site, which leads to disturbance of contacts in the active site of enzymes.



**Figure 2.** Influence of divalent cations ( $Mg^{2+}$ ) on the AP-endonuclease activity of enzymes. Accumulation of the reaction product **P** from F-substrate **S** as determined by PAGE (A). Dependences of the cleavage efficacy on  $Mg^{2+}$  concentration (B). Errors were calculated by average each data point over at least three replicates.

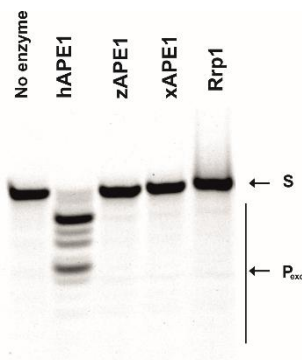
### 3.2 3'-5' Exonuclease activity

Despite very similar activity of APE1-like endonucleases toward main biological substrate containing an abasic site, the ability of homologues enzymes to 3'-5' exonucleosidic degradation of the DNA was significantly differs. As shown in Fig. 3A only hAPE1 possesses pronounced 3'-5' exonuclease activity. Therefore the dependences of 3'-5' exonuclease cleavage of non-damaged DNA on  $MgCl_2$  or  $KCl$  concentrations were obtained only for hAPE1 (Fig. 3B). Interestingly to note, that the broad bell-shaped  $K^+$  dependence (Fig. 3C) and maximal activation by 1.0 mM  $Mg^{2+}$  ions (Fig. 3D) was very similar between AP-endonuclease (Fig. 1 and 2) and 3'-5' exonuclease (Fig. 3) activities of hAPE1. It was previously shown by Wang Y. et al that zAPE1 has a very weak 3'-5' exonuclease activity, which is ~100-fold lesser than the AP-

endonuclease activity of this enzyme [46]. Moreover, up to date there are no studies concerning the 3'-5' exonuclease activity of the xAPE1 enzyme. Taking together, the barely noticeable 3'-5' 3'-5' exonucleosidic degradation of the model DNA by these enzymes is consistent with literature data and, most likely, is not associated with the plasmid constructions used to produce these enzymes in *E. coli* expression system.

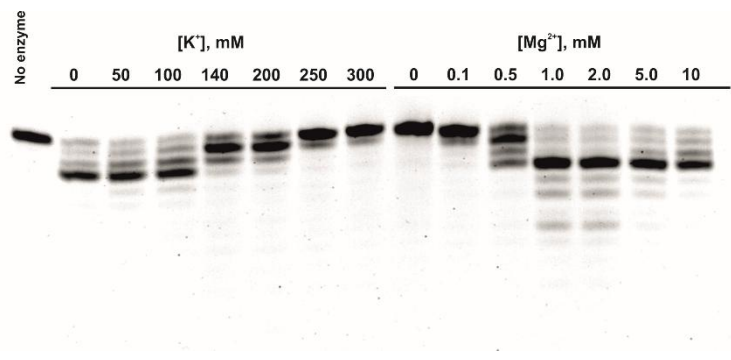
On the other hand it was unexpectedly found that the Rrp1 enzyme exhibits no significant 3'-5' exonuclease activity, as it was demonstrated 30 years earlier [47] when initially was reported that enzyme is able to implement 3'-5' exonucleosidic degradation of the DNA. Nevertheless, it was shown later that 3'-5' exonuclease activity is dependent on the DNA context [48]. It also has been demonstrated [49] that, unlike the hAPE1, the Rrp1's 3'-5' exonuclease activity depends on the presence in its structure of the full-sized N-terminal domain. Considering the fact that Rrp1 possesses the largest N-terminal domain compared to other tested AP-endonucleases, it could be assumed that structural features of this non-catalytic domain in the full-sized enzyme produced in *E. coli* expression system could affect the 3'-5' exonuclease activity of Rrp1.

A

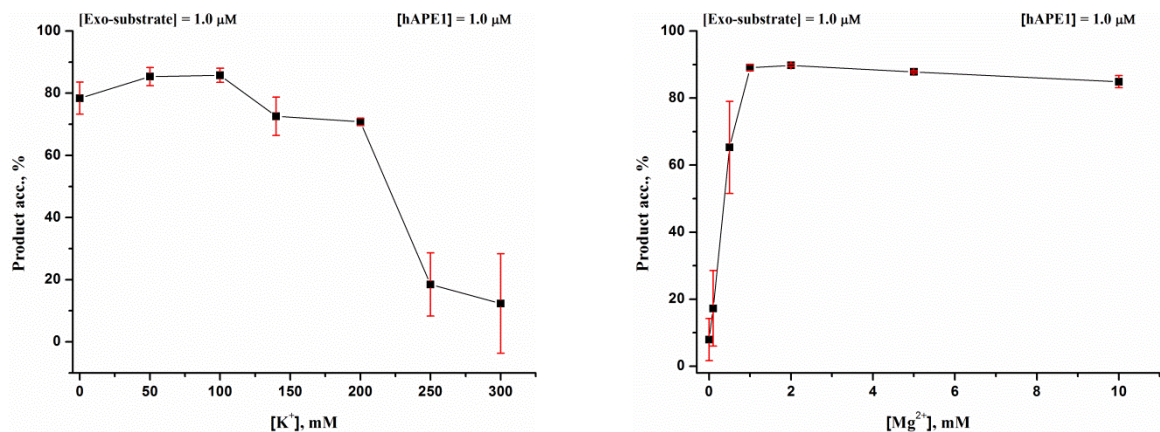


C

B



D



**Figure 3.** PAGE analysis of 3'-5' exonuclease activity of APE1-like enzymes. Comparison of 3'-5' exonucleosidic degradation of recessed DNA (A). Effect of K<sup>+</sup> or Mg<sup>2+</sup> ions on the 3'-5' exonuclease DNA cleavage in the course of interaction with hAPE1. Dependences of the cleavage efficacy of hAPE1 on K<sup>+</sup> ions (B) or Mg<sup>2+</sup> ions (C) concentration. Errors were calculated by average each data point over at least three replicates.

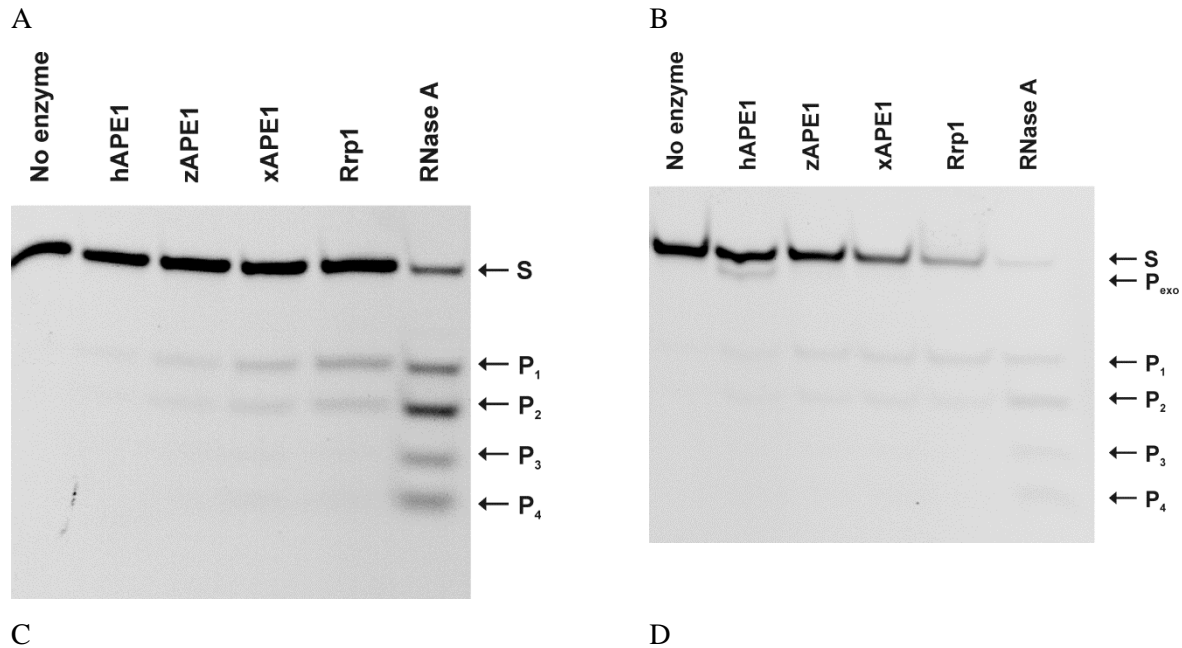
### 3.3 Endoribonuclease activity

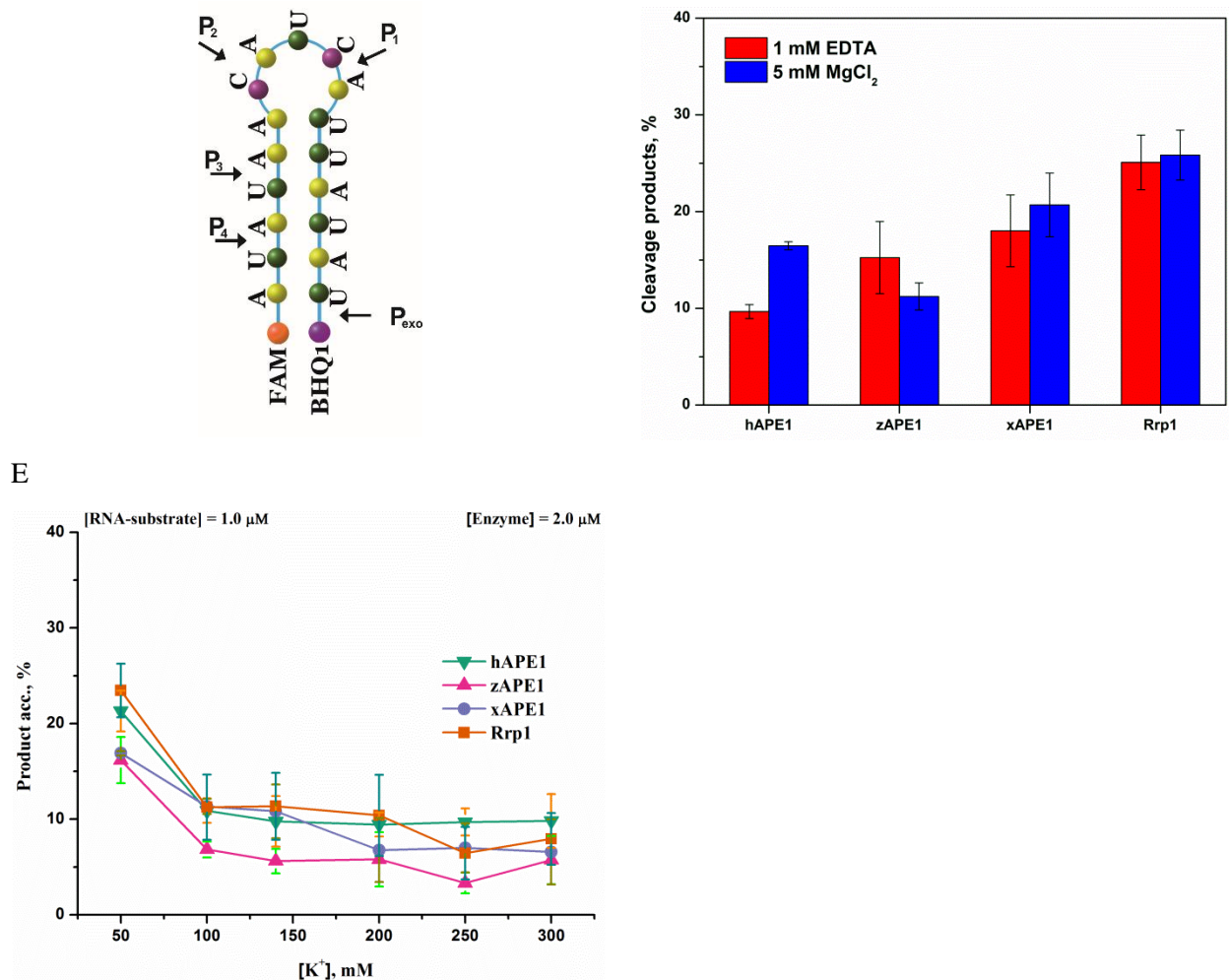
The key difference between the catalytic mechanisms of phosphodiester bond hydrolysis in RNA- and DNA-substrates is that the reaction yields different products: 3'-PO<sub>4</sub><sup>2-</sup> for RNA and 3'-OH for DNA [50]. Moreover, hAPE1 does not need divalent metal ions to exhibit its endoribonuclease activity [50,51], supporting difference of catalytic mechanism of DNA and RNA cleavage. Undamaged RNA fragments are preferentially cleaved at the phosphodiester bond between the UA, UG, and CA dinucleotides in single-stranded sequences or weakly paired RNA regions [51]. As recently was revealed [52–54], short hairpin folded RNA-substrate exhibits the best efficacy to be cleaved among the broad set of native RNA tested in the endoribonuclease cleavage assay with hAPE1. Interestingly to note that the ability to hydrolyze RNA was shown with respect to microRNA, c-myc mRNA, CD44 mRNA, and RNA components of the SARS coronavirus [20,55]. It was suggested [20] that endoribonuclease hydrolysis of mRNA may be one of the important function of the hAPE1 in the cytoplasm.

Therefore, a comparative analysis of the endoribonuclease hydrolysis of model RNA-substrate by the four APE1-like enzymes was performed. The interaction of APE1-like enzymes with model RNA substrate was analyzed both in the absence of divalent metal ions (Fig. 4A) and in the presence of Mg<sup>2+</sup> ions (Fig. 4B). As it was found by PAGE analysis four sites of

endoribonuclease cleavage are presented in the model RNA-substrates, which are shown by arrows in Fig. 4C. Interestingly to note, that only during interaction of hAPE1 with RNA-substrate in a buffer solution supplemented by 5 mM MgCl<sub>2</sub> the additional 3'-5' exonuclease product P<sub>exo</sub> was detected (Fig. 4B), supporting that 3'-5' exonuclease activity of hAPE1 toward RNA-substrate is most efficient among all tested enzymes.

The relative activities of APE1-like enzymes in relation to RNA-substrate in the presence of EDTA and Mg<sup>2+</sup> ions was determined (Fig. 4D). These data clearly indicate that endoribonuclease activity of enzymes slightly increases in the order hAPE1 ~ zAPE1 ≤ xAPE1 ≤ Rrp1. On the other hand, dependences of the RNA cleavage efficacy on concentration of K<sup>+</sup> ions were similar for all tested enzymes and significantly differ from the dependence of AP-endonuclease and 3'-5' exonuclease activities. Indeed, increase of KCl concentration over 100 mM leads to decrease of RNA cleavage efficiency at minimal level ~10%, whereas in the case of DNA cleavage efficiency reached this level at 250 mM concentration of KCl.





**Figure 4.** The efficiency of cleavage of RNA-substrate by APE1-like enzymes. PAGE analysis of the reaction products in the presence of 1.0 mM EDTA (A) or 5.0 mM MgCl<sub>2</sub> (B). Positions of the hydrolyzed nucleotides are pointed out by arrows as revealed by RNase A cleavage (C). Comparison of the efficacy of cleavage of RNA-substrates by APE1-like enzymes (D). Dependences of the RNA cleavage efficacy on K<sup>+</sup> ions concentration (E). Errors were calculated by average each data point over at least three replicates. [Enzyme] = 2.0 μM, [RNA] = 1.0 μM, reaction time = 1 h.

#### 4. Discussion

Despite significant achievements in the understanding of functional properties of AP endonucleases, the question how a particular enzyme controls their activity toward various substrates remains unanswered. It was commonly thought that the main biological function of AP-endonucleases was the hydrolytic cleavage at the 5' AP-site and subsequent formation of



single strand break with a 5'-deoxyribosephosphate and a 3'-hydroxyl group [5,56]. Indeed, obtained in the present study data on the metal-dependence of AP-endonuclease activity revealed wide bell-shaped dependence of AP-endonuclease activity on KCl concentration as well as strong activation of enzymes by 1.0 mM MgCl<sub>2</sub>. Such very similar behavior for all tested enzymes suggests that the main biological function is highly conserved for evolutionary distant species.

AP-endonucleases recognize non-damaged nucleotides when catalyze 3'-5' exonuclease [40,57] and endoribonuclease [50] cleavage of DNA and RNA, respectively. Obtained data demonstrate that the efficiency of 3'-5' exonuclease activity was significantly higher for hAPE1 in comparison with other tested enzymes. Moreover, the effect of metal ions on the 3'-5' exonuclease activity of hAPE1 was very similar to that of AP-endonuclease activity. These data suggest that 3'-5' exonuclease activity of hAPE1 could be important biological function in mammals. Moreover, it should be noted that mammalian cells possess two APE1-like enzymes (APE1 and APE2), which have fully conserved active site amino acids [18,22,58]. Human enzyme hAPE1 has a highly efficient AP-endonuclease activity, however, its 3'-end cleansing including 3'-5' exonuclease activity is substantially low. Contrary to hAPE1, the 3'-5' exonuclease activity of hAPE2 is higher than its AP-endonuclease activity [59]. Moreover, several other APE1-like AP-endonucleases such as MtbXthA from *Mycobacterium tuberculosis*, NApe and NExo from *Neisseria meningitides* and APE1L from *Arabidopsis thaliana* completely lack the ability to process base-damaged nucleotides despite common architecture of catalytic site [60–62]. These facts allow to assume that efficiency of 3'-5' exonuclease activity is not controlled by network of conserved catalytic active site amino acids, but rather depend on some additional factors, such as, probably, significantly different N-terminal domains of APE1-like enzymes or the global plasticity of active site pocket. This conclusion is in agreement with data [13] on pre-steady-state analysis of the interaction of the tested APE1-like enzymes with DNA-substrates containing various base-damaged nucleotides. Because the differences in rates of DNA substrates' binding do not lead to significant differences in the cleavage efficiency of DNA containing a damaged base, therefore it is reasonable to conclude that the cleavage efficacy is related to the fine conformational tuning inside the active site.

On the other hand, the N-terminal domain of tested APE1-like enzymes differs significantly in size and consistence. Previous studies [10,11,63] revealed that a loss of the N-

terminal domain in hAPE1 affects both the rate of formation and the stability of the initial complex in the cases of base-damaged DNA cleavage and 3'-5' exonuclease activity. Moreover, analysis of N-terminally truncated hAPE1 variants has shown that this lysine-rich region can act as a stabilizer for binding to nucleic acids [64,65]. By contrast, this domain does not influence the main AP-endonuclease function of hAPE1 [11,66,67] in the case of abasic DNA cleavage.

It was found that all tested APE1-like enzymes possess endoribonuclease activity in the absence of cofactor  $Mg^{2+}$  ions, as was previously reported for hAPE1 [54], suggesting the common mechanism of RNA cleavage. Obtained data showed that endoribonuclease activity of enzymes increases in the order hAPE1 ~ zAPE1  $\leq$  xAPE1  $\leq$  Rrp1 with strong inhibition by 100 mM KCl concentration. These data allow to assume the possible important role of N-terminal domain of enzymes in the interaction with RNA-substrate.

Taken together, in the present study we have compared the effects of mono- and bivalent metal ions on three different types of activities of APE1-like enzymes: AP-endonuclease, 3'-5' exonuclease and endoribonuclease activities. For this purpose cleavage efficiency of model damaged and non-damaged DNA- and RNA-substrates have been analyzed. It was shown that despite conservative catalytic amino acids of active site and very similar behavior of main AP-endonuclease activity, these enzymes demonstrate significantly different ability in 3'-5' exonucleosidic degradation of non-damaged DNA. The most efficient 3'-5' exonucleosidic degradation of model non-damaged DNA was catalyzed by hAPE1, suggesting biological importance of this function in mammals. On the other hand, obtained data indicate that variety in the endoribonuclease activity could be associated with the action of N-terminal domain, which could bind nucleic acids through electrostatic interactions. The interactions with N-terminal domain in the case of RNA binding is confirmed by high sensitivity to monovalent  $K^+$  ions in comparison with other enzyme activities.

**Conflicts of Interest:** *The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.*

### **Author Contributions**



Conceptualization, N.A.K.; methodology, A.T.D., A.A.K., D.S.N.; software, A.T.D., A.A.K.; validation, A.A.I., M.S., N.A.K., and O.S.F.; formal analysis, A.T.D., A.A.K.; investigation, A.T.D., A.A.K., D.S.N.; resources, A.A.I., M.S., N.A.K., and O.S.F.; data curation, A.A.I., M.S., N.A.K., and O.S.F.; writing—original draft preparation, A.T.D., A.A.K., and N.A.K.; writing—review and editing, A.A.I., M.S., N.A.K. and O.S.F.; visualization, A.T.D., A.A.K.; supervision, N.A.K.; project administration, N.A.K.; funding acquisition, A.A.I., M.S., A.A.K. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported partially by a Russian-Government-funded project (no. 121031300041-4), by Electricité de France (RB 2020-02 and RB 2021-05, to M.S.), by the French National Research Agency (ANR-18-CE44-0008), and Fondation ARC (PJA-2021060003796) to A.A.I. The part of the work involving the analysis of 3'-5' exonuclease and endoribonuclease activities was specifically funded by the Russian Science Foundation, grant no. 21-64-00017. A.T.D. was supported by Ph.D. grant no. 20-34-90008 from the Russian Foundation for Basic Research.

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