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3′-5′ Exonuclease Activity of Human Apurinic/Apyrimidinic Endonuclease 1 towards DNAs Containing dNMP and Their Modified Analogs at the 3′ End of Single Strand DNA Break

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Abstract—Human DNA apurinic/apyrimidinic (AP-) endonuclease 1 (APE1) is involved in the base excision repair (BER) pathway. The enzyme hydrolyzes DNA from the 5′ side of the AP site. In addition to endonuclease activity, APE1 also possesses other slight activities including 3′-5′ exonuclease activity. The latter is preferentially exhibited towards mispaired (non-canonical) nucleotides, this being the reason why APE1 is considered as a proofreading enzyme correcting the misincorporations introduced by DNA polymerase β. We have studied 3′-5′ exonuclease activity of APE1 towards dCMP and dTMP residues and modified dCMP analogs with photoreactive groups at the 3′ end of the nicked DNA. Photoreactive dNMP residues were incorporated at the 3′ end of the lesion using DNA polymerase β and photoreactive dNTPs. The dependence of exonuclease activity on the "canonicity" of the base pair formed by dNMP flanking the nick at the 3′ end, on the nature of the group flanking the nick at the 5′ end, and on the reaction conditions has been determined. Optimal reaction conditions for the 3′-5′ exonuclease hydrolysis reaction catalyzed by APE1 *in vitro* have been established, and conditions when photoreactive residues are not removed by APE1 have been chosen. These reaction conditions are suitable for using photoreactive nicked DNAs bearing 3′-photoreactive dNMP residues for photoaffinity labeling of proteins in cellular/nuclear extracts and model APE1-containing systems. We recommend using FAPdCTP for photoaffinity modification in APE1-containing systems because the FAPdCMP residue is less prone to exonuclease degradation, in contrast to FABOdCTP, which is not recommended.

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Key words: APE1 exonuclease activity, modified photoreactive dNTP analog

Human apurinic/apyrimidinic (AP-) endonuclease 1 (APE1) is a multifunctional enzyme. APE1 is one of the major components of the base excision repair (BER) pathway, correcting DNA with damaged heterocyclic bases and spontaneously emerging AP sites. APE1 cleaves the DNA sugar-phosphate backbone 5′ to the AP site, forming a nick flanked by the hydroxyl group at the 3′ end and deoxyribophosphate (dRP) at the 5′ end. The enzyme also possesses weak 3′ phosphodiesterase, 3′-5′ exonuclease, 3′ phosphatase, and RNase H activities [1]. Irrespective of DNA repair function, APE1 was identified as a redox factor, Ref-1 [2, 3].

The data on the biological significance of 3′-5′ exonuclease activity of APE1 are controversial. Previously this activity was regarded as insignificant since its efficiency was several (2-4) orders of magnitude lower than AP-endonuclease activity [4]. However, interest in exonuclease activity has recently reappeared after APE1 was identified as the exonuclease which effectively cleaves some of the modified nucleotide analogs (for instance, 3′-azido-3′-deoxythymidine, 2′,3′-didehydro-2′,3′-dide-

Abbreviations: BER) base excision repair; APE1) apurinic/apyrimidinic exonuclease 1 (human); AP site) apurinic/apyrimidinic site; β-pol) DNA polymerase β; NIR) nucleotide incision repair; ssDNA) single strand DNA; dsDNA) double strand DNA.

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oxythymidine, and β-L-dioxolane-cytidine [5, 6]) at the 3′ end of a single strand DNA break. Nucleosides corresponding to the listed dNMP analogs are used in anti-HIV and anti-cancer therapy, as well as for treatment of several viral diseases. The same group of researchers found that APE1 is capable of more effective cleavage of mismatched (non-canonically paired) nucleotides compared to correctly paired nucleotides [5]. Based on this observation, it has been assumed that APE1 plays a correcting role in BER, i.e., is a factor that enhances the fidelity of the DNA repair process. It should be noted that the main DNA polymerase of base excision repair, DNA polymerase β (β-pol), displays low fidelity in DNA synthesis due to the lack of proofreading 3′-5′ exonuclease activity. At the same time, the fidelity of DNA repair synthesis *in vivo* is noticeably higher than that *in vitro* [7]; therefore, elucidation of the factors that enhance the fidelity of this process is of great interest. Comparative analysis of the publications on the proofreading role of APE1 revealed a significant difference between the data obtained by different investigators, both regarding error correction and factors influencing the level of APE1 exonuclease activity (for example, the nature of DNA duplex, reaction conditions) [3, 5, 8-10]. These facts make it impossible to unambiguously state that 3′-5′ exonuclease activity is efficiently displayed in real biological systems. An important trend is the search for conditions favoring this activity and being similar to the optimum of major endonuclease function of APE1. A number of recent publications have demonstrated that repair of DNA lesions is possible in the case of pyrimidine nucleotides with oxidized heterocyclic base, when repair occurs without DNA glycosylases. The authors have shown that under certain reaction conditions, APE1 is able to cleave DNA at the 5′ end of the lesions without the formation of an intermediate AP site, in contrast to DNA glycosylases. Such a mechanism was called nucleotide incision repair (NIR) and is an alternative pathway for correction of oxidative DNA damage [11]. Moreover, it was found that APE1 can cleave DNA in the vicinity of the AP site not only in the double stranded (dsDNA), but also in single strand (ssDNA) substrates [12]. It is interesting to note that optimal conditions for the reactions of exonuclease cleavage, NIR, and hydrolysis of AP site in ssDNA were similar to each other and different from the optimal conditions for the major endonuclease reaction [11, 12]. In this connection, a comparative analysis of optimal conditions for these additional APE1 functions towards native and modified dNMP *in vitro* is of interest.

This work focuses on determination of APE1 3′-5′ exonuclease activity towards DNA substrates with photoreactive dCMP analogs positioned at the 3′ end of the nick and used for photoaffinity modification of proteins and DNA in comparison with native dNMP. The structure of analogs in nucleoside-5′-triphosphate form and their abbreviations are given in Fig. 1. The derivatives of pyrimidine nucleoside-5′-triphosphates with large aromatic substituents in the heterocyclic base are effective substrates for all studied DNA polymerases [13, 14]. This property is used for the synthesis of primers containing photoreactive groups at the 3′ end followed by their subsequent use for protein affinity modification. Investigation of cleavage efficiency for these analogs is important for their use in the reconstructed systems as well as cell and nuclear extracts containing APE1. This work uses several photoreactive dCTP analogs containing photoreactive substituents at exo-N-position of cytosine. Structure of the spacer arm linking the photoreactive group to the base at the same position can induce a shift in tautomeric equilibrium from amino- to imino-form, altering the properties of substrate analog in the reaction of oligonucleotide elongation by various DNA polymerases from dCTP-like to dTTP-like [13]. Thus, DNA substrates containing these photoreactive analogs of dNMP residues can be considered not only as modified dNMP, but also as models for matched and mismatched nucleotides. We have determined the cleavage efficiency (as percentage of exonuclease reaction product compared to total DNA amount) of dNMP analogs inserted at the 3′ end of single strand break in different DNA substrates.

MATERIALS AND METHODS

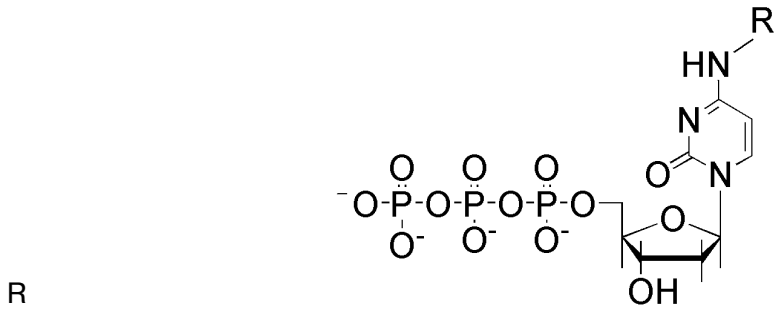
The following chemicals were used in this work: T4 polynucleotide kinase, rat β-pol purified according to [14], human APE1 purified as described in [8] (Laboratory of Bioorganic Enzyme Chemistry, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences), T4 DNA ligase, [γ-^{32}P]ATP (Biosan, Russia); BSA (BioLabs, UK); non-radioactive dATP and ATP (Promega, USA); chemicals for electrophoresis, Tris, $MgCl_2$, dyes Coomassie G-250, xylene cyanole, and bromophenol blue (Sigma, USA); Hepes (Fluka, Switzerland). Other chemicals were of analytical grade and purchased from Russian suppliers. Plasmid vectors were kindly provided by Dr. S. H. Wilson (NIEHS, NC, USA).

Synthetic oligonucleotides were acquired from Genset (France) or the oligonucleotide synthesis group (ICBFM, SB RAS). The following oligonucleotides were used:

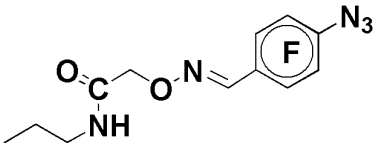
TA: 5′-GGA AGA CCC TGA CGT TAC CCA ACT TAA TCG CC-3′

TG: 5′-GGA AGA CCC TGA CGT TGC CCA ACT TAA TCG CC-3′

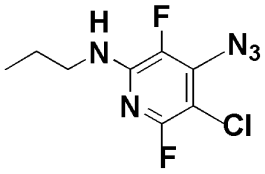
P: 5′-GGC GAT TAA GTT GGG-3′



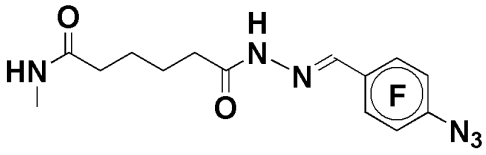
FABO-dCTP – N4-[(4-azido-2,3,5,6-tetrafluorobenzylidene-aminooxy)-butyloxy]-2′-deoxycytidine 5′-triphosphate (lithium salt)



FABC-dCTP – exo-N-[2-(4-azido-2,3,5,6-tetrafluorobenzylidene-aminooxymethyl-carbamoyl)ethyl]-2′-deoxycytidine 5′-triphosphate (lithium salt)



FAP-dCTP – exo-N-[2-N-(N-(4-azido-2,5-difluoro-3-chloropyridin-6-yl)-3-aminopropionyl)-aminoethyl]-2′-deoxycytidine 5′-triphosphate (lithium salt)



FABG-dCTP – exo-N-[4-(4-azido-2,3,5,6-tetrafluorobenzylidene-hydrazinocarbonyl)-butylcarbamoyl]-2′-deoxycytidine 5′-triphosphate (lithium salt)

Fig. 1. Structures of photoreactive dCTP analogs.

PT: 5′-GGC GAT TAA GTT GGG T-3′

PC: 5′-GGC GAT TAA GTT GGG C-3′

BP1: 5′-pAAC GTC AGG GTC TTC C-3′

BP2: 5′-pFAAC GTC AGG GTC TTC C-3′

BP3: 5′-AAC GTC AGG GTC TTC C-3′.

^{32}P-Labeling of P, PT, and PC oligonucleotides at 5′ end was performed using T4 polynucleotide kinase; labeled PT and PC oligonucleotides were purified by electrophoresis in denaturing polyacrylamide gel in the presence of 7 M urea [15], and further used to prepare ^{32}P-labeled DNA duplexes (Table 1).

Synthesis of DNA substrates containing photoreactive dCMP analogs at the 3′ end of the primer using β-pol. The reaction mixture contained the following standard components: 50 mM Tris (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$ (buffer 1, Table 2), as well as 0.6 μM DNA duplex (DNA1 – TG + P) with ^{32}P-labeled P oligonucleotide, 80 μM dCTP analog, and 3 μM β-pol. Elongation products were analyzed and purified by electrophoresis in denaturing polyacrylamide gel with 7 M urea [15], and the resulting ^{32}P-labeled photoreactive oligonucleotide products were used to prepare DNA duplexes. The structures and abbreviations for the resulting DNA duplexes are given in Table 1.

Ligation of nick containing photoreactive dNMP analog at the 3′ end. Reaction mixtures (volume 10 μl) contained 0.1 μM DNA with photoreactive dNMP analog at

Table 1. DNA structures and sequences

DNA structure and sequence	Abbreviation
5'- GG-CGA-TTA-AGT-TGG-G-3' 3'- CC-GCT-AAT-TCA-ACC-C - **G** - T-TGC-AGT-CCC-AGA-AGG -5'	DNA1
p-5' 5'- GG-CGA-TTA-AGT-TGG-G-**X** A-ACG-TCA-GGG-TCT-TCC -3' 3'- CC-GCT-AAT-TCA-ACC-C - **G** - T-TGC-AGT-CCC-AGA-AGG -5'	XG (p)
pF-5' 5'- GG-CGA-TTA-AGT-TGG-G-**X** A-ACG-TCA-GGG-TCT-TCC -3' 3'- CC-GCT-AAT-TCA-ACC-C - **G** - T-TGC-AGT-CCC-AGA-AGG -5'	XG (pF)
5'- GG-CGA-TTA-AGT-TGG-G-**X** 5'A-ACG-TCA-GGG-TCT-TCC -3' 3'- CC-GCT-AAT-TCA-ACC-C - **G** - T-TGC-AGT-CCC-AGA-AGG -5'	XG (OH)
p-5' 5'- GG-CGA-TTA-AGT-TGG-G-**X** A-ACG-TCA-GGG-TCT-TCC -3' 3'- CC-GCT-AAT-TCA-ACC-C - **A** - T-TGC-AGT-CCC-AGA-AGG -5'	XA (p)
pF-5' 5'- GG-CGA-TTA-AGT-TGG-G-**X** A-ACG-TCA-GGG-TCT-TCC -3' 3'- CC-GCT-AAT-TCA-ACC-C - **A** - T-TGC-AGT-CCC-AGA-AGG -5'	XA (pF)
5'- GG-CGA-TTA-AGT-TGG-G-**X** 5'A-ACG-TCA-GGG-TCT-TCC -3' 3'- CC-GCT-AAT-TCA-ACC-C - **A** - T-TGC-AGT-CCC-AGA-AGG -5'	XA (OH)

Note: X = C (dCMP), T (dTMP), FABOdCMP, FABCdCMP, FABGdCMP, FAPdCMP.

Table 2. Conditions for APE1 exonuclease activity

Buffer	Buffer component	pH	BSA, 0.1 mg/ml	$MgCl_2$, mM	KCl, mM
1	Tris	8.0	—	10	50
2	Tris	6.8	—	10	50
3	Tris	6.8	—	0.5	25
4	Tris	6.8	+	0.5	25
5	Hepes	6.8	—	0.5	25
6	Hepes	6.8	+	0.5	25

the 3′ end of the nick (XG(p) or XA(p), Table 1), 2.5 units of T4 DNA ligase, and 1 mM ATP. Reaction was performed in buffer 1 (Table 2) for 60 min at 21°C. Reaction products were separated by electrophoresis in 20% denaturing polyacrylamide gel [15].

Incorporation of native dNTP after photoreactive analog at the 3′ end of the nick by β-pol. Reaction mixtures contained 0.1 μM DNA with photoreactive dNMP analog at the 3′ end of the nick (XG(p) or XA(p), Table 1), 2 μM of β-pol, and 20 μM dATP. Reaction was performed in buffer 1 (Table 2) for 30 min at 37°C. The products were separated by electrophoresis in 20% denaturing polyacrylamide gel [15].

Exonuclease activity of APE1. Reaction mixtures contained 0.02 μM DNA, various concentrations of APE1 as well as buffer components: 50 mM Tris or 20 mM Hepes (pH values for various buffers are indicated in Table 2), $MgCl_2$ and KCl; buffers 4 and 6 also contained BSA. The mixture was incubated for 30 min at 37°C. Reaction products were separated by electrophoresis in 20% denaturing polyacrylamide gel [15].

RESULTS

Photoreactive analogs as models for native dNMP in matched and mismatched nucleotide base pairs. Considering that APE1 removes mismatched nucleotide residues more effectively [5, 9, 10] and that the analogs in this study can behave like dCTP and dTTP [13], it was interesting to

investigate the influence of the substituent in the heterocyclic base on exonuclease activity of APE1. Two types of DNA templates were used to prepare DNA duplexes: those with dAMP (A) and dGMP (G) opposite to the photoreactive analog, with the aim of determining the correlation between the displayed exonuclease activity and ability of dNMP analog to form a matched pair with the corresponding base in the template. Several approaches were applied for the estimation of "canonicity" of the pair between modified analog and dNMP in the template (Fig. 2):

– incorporation of native dNTP after modified photoreactive analog at the 3′ end of the nick, since it is known that mismatched dNMP residues at the 3′ end are not efficiently elongated by the enzyme [16] (Fig. 2a);

– dependence of ligation efficiency of the nick containing photoreactive analog at the 3′ end on the type of nucleotide (A or G) in the opposite strand; because the formation of mismatch at the 3′ end of the nick decreases the efficiency of sugar-phosphate backbone repair by T4 DNA ligase [17] (Fig. 2b).

The data on efficiency of native dNTP elongation after the photoreactive analog show good correlation with ligation data as well as with substrate properties of dCTP residues in the elongation reaction catalyzed by β-pol [13, 14, 18]. Moreover, these data are in agreement with the data on dependence of APE1 exonuclease activity on the nature of nucleotide (A or G) opposite to the photoreactive analog (Fig. 2c). Table 3 summarizes the data on APE1 exonuclease activity towards all DNA duplexes containing a nick and used in this work (Fig. 2). Thus, FABGdCMP, FABCdCMP, and FAPdCMP are rather dCMP (C) than dTMP (T) analogs, whereas FABOdCMP is equally C and T analog.

These photoreactive dNMP are analogs of dCMP or dTMP, so we further investigated efficiency of exonuclease cleavage of dCMP and dTMP at the 3′ end of the primer in the nick by APE1 for comparison with the results obtained for 3′-5′ exonuclease activity of APE1 towards the modified DNA duplexes. As a control for DNA substrates containing modified residues, we used

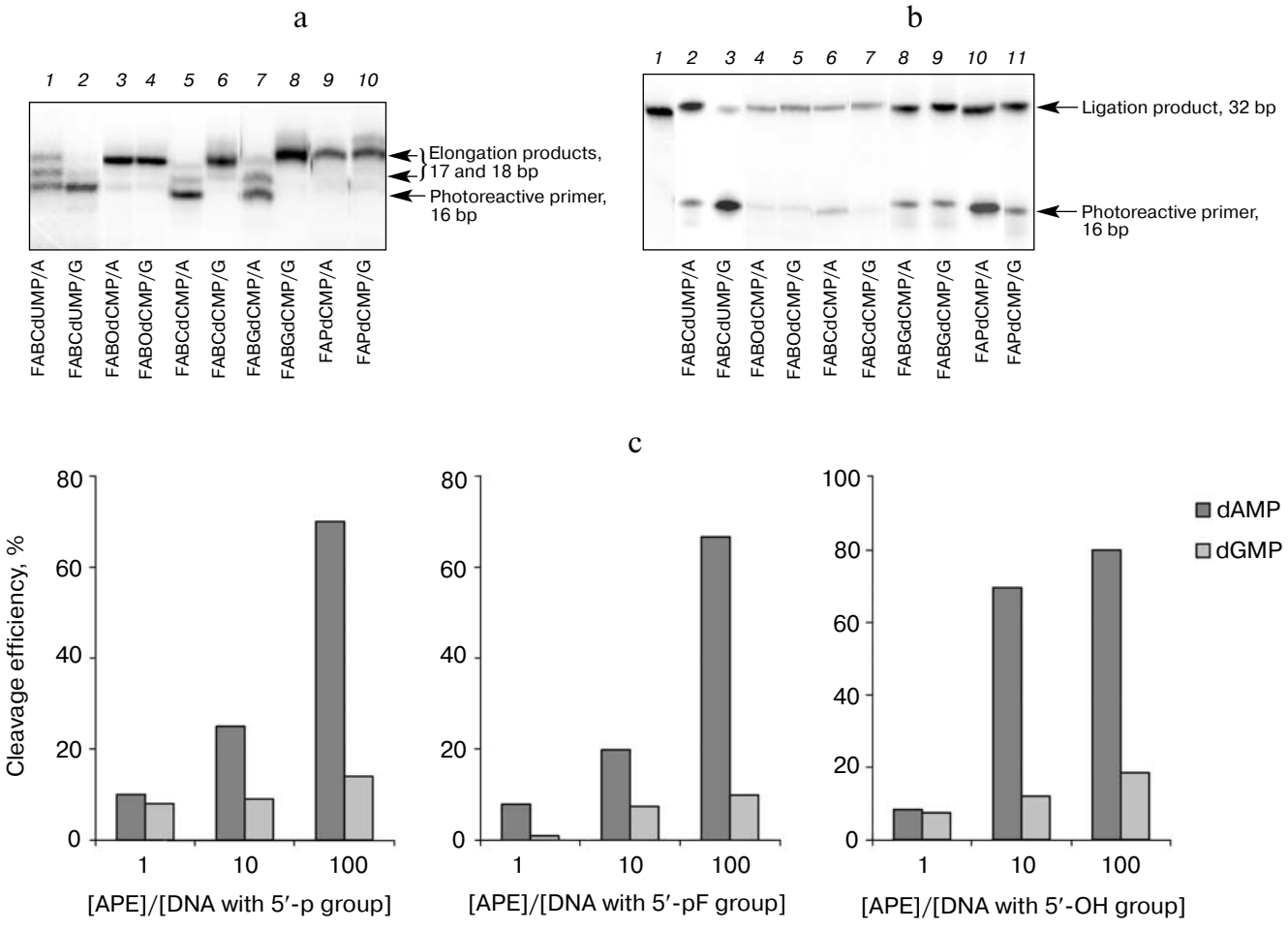


Fig. 2. Estimation of "canonicity" of the nucleotide pair formed by a modified dNMP analog and a template. a) Incorporation of native dNTP after a photoreactive analog at the 3′ end of the nick using β-pol. b) Ligation of the nick containing photoreactive dNMP analog at the 3′ end. c) Exonuclease activity of APE1 (FABCdCMP opposite to A or G in the template).

Table 3. APE1 3′-5′ exonuclease activity, efficiency of the removal of dNMP from the 3′ end of single strand break expressed as percent of the exonuclease reaction product from total amount of DNA substrate

3′-dNMP	5′-group	APE1/DNA	Buffer 1		Buffer 6	
			opposite to A in the template	opposite to G in the template	opposite to A in the template	opposite to G in the template
1	2	3	4	5	6	7
FABOdCMP	p	1	18.8	16	92	86.5
		2	70	20.4	91	91
		3	90	60	94	93
	pF	1	16	15	87	90
		2	75	42	90	91
		3	88	90	93	93
	OH	1	30	28	92	92
		2	86	75	90	91
		3	92	89	92	93
FABCdCMP	p	1	10	<10	90.3	45
		2	25	<10	91.6	81
		3	70	14	92	92
	pF	1	10	<10	31.5	<10
		2	20	—	48.5	13
		3	67	10	83	20
	OH	1	11	<10	85	84
		2	70	12	90	89.5
		3	80	18.5	93	92
FABGdCMP	p	1	14	8	82.3	40.7
		2	30	10	87.6	76.9
		3	55	22	92	91.9
	pF	1	10	<10	21.3	10.8
		2	25	9.5	37.5	12.5
		3	45	14	70	16.5
	OH	1	35	15	81	85
		2	74	57	87	89.8
		3	85	78	91	92.7
FAPdCMP	p	1	3.6	<10	90.7	57.8
		2	18.9	—	93	90.7
		3	82.2	10	94.7	93.9
	pF	1	4.7	<10	43.8	3.6
		2	48.4	—	83.5	5.4
		3	83.8	<10	92	21.1
	OH	1	14	<10	87.2	77.8
		2	80.6	13.5	89.1	90.5
		3	85.6	51.6	94.7	94.3
dCMP	p	1	15	<10	73	25
		2	21	15	85	50
		3	30	20	92	88

Table 3. (Contd.)

1	2	3	4	5	6	7
	pF	1	<10	<10	15	<10
		2	16	14	51	20
		3	22	17	80	41
	OH	1	18	17	79	29
		2	53	22	85	77
		3	78	36	94	90
dTMP	p	1	10	15	30	50
		2	16	18	70	84
		3	25	36	90	92
	pF	1	12	15	16	15
		2	15	17	20	42
		3	17	26	45	85
	OH	1	11	13	66	80
		2	23	56	74	90
		3	37	80	91	93

Note: For buffer 1: 1, 2, and 3 stand for 1, 10, 100; for buffer 6: 0.25, 1, and 4. Experimental error did not exceed 10%.

four types of DNA substrates; two of them contained a matched pair at the 3′ end of the primer (CG and TA, the first letter being the nucleotide attacked by APE1, the second letter the nucleotide in the opposite strand), and the other two contained mismatched pair at the 3′ end of the primer (TG or CA). Cleavage efficiency of native dNMP analogs by APE1 decreased in the order CA > TG > TA ≥ CG (Table 3).

Dependence of APE1 3′-5′ exonuclease activity on structural characteristics of the oligonucleotide at the 5′ end of the nick. Besides the investigation of dependence of exonuclease cleavage on the presence of A or G in the template, we also studied the influence of the flanking group at the 5′ end of the nick. Three types of blocking primers (BP) were used: BP containing phosphate, without phosphate (hydroxyl), and with tetrahydrofuran

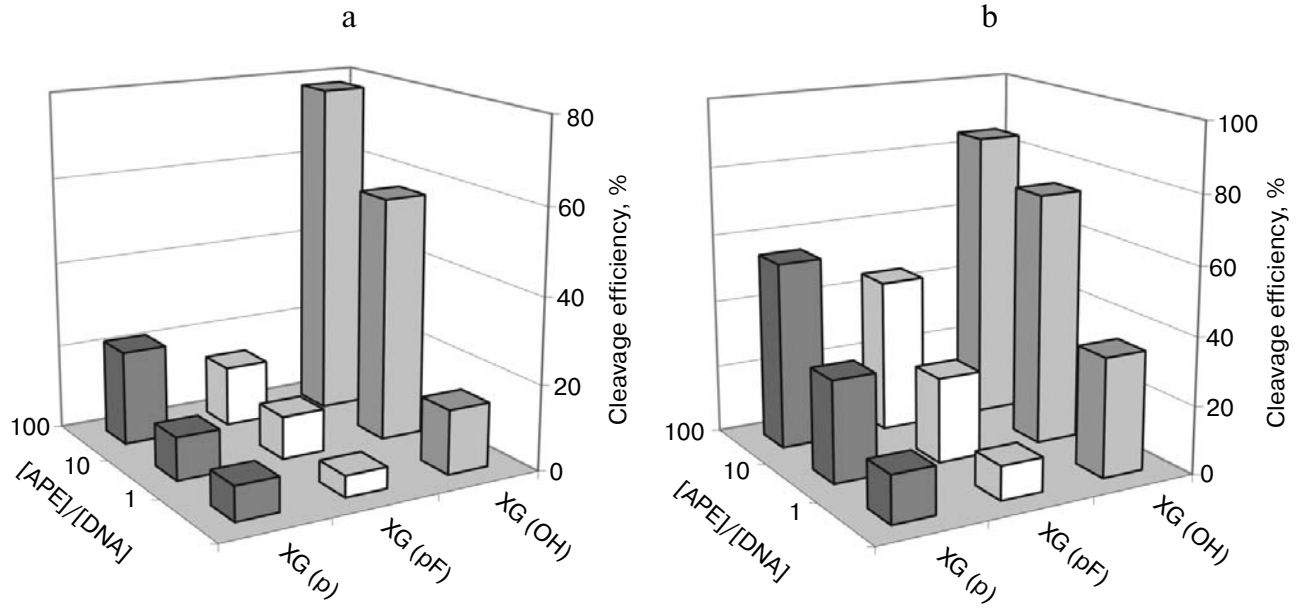


Fig. 3. APE1 3′-5′ exonuclease activity towards DNA substrates with 5′-p, 5′-pF, and 5′-OH groups in the nick using model DNA duplexes containing FABGdCMP at the 3′ end of the nick in buffer 1: a) opposite to dGMP of the template; b) opposite to dAMP of the template.

Table 4. Reaction conditions for different APE1 activities

Activity, literature source		pH		[KCl/NaCl], mM		[$MgCl_2$], mM (in the presence of KCl)
		range	optimum	range	optimum	
Endo-	[20]	6.7-9.0	7.5-7.8	–	–	–
	[11]	–	7.8-8.2	25-200	–	–
	[3]	6.2->8.0	7.7	up to 300	120	2
					20	12.5
	[12]	7.0-7.5	–	–	50	10
				–	200	2
Exo-	[3]	6.2-7.9	7.4	up to 100	–	2
NIR	[11]	–	6.4-6.8	up to 50	–	–
Endo-ssDNA	[12]	7.0-7.5	–	–	⩽50	0.5-2

phosphate – a sugar-phosphate that mimics the deoxyribophosphate at the 5′ end. For native dNMP and all investigated analogs except for FABOdCMP, the efficiency of dNMP cleavage decreased in the order: hydroxyl (OH) > phosphate (p) > tetrahydrofuran phosphate (pF) (Fig. 3 and Table 3).

Dependence of 3′-5′ exonuclease activity of APE1 on reaction conditions. As shown previously [3, 19], the level of APE1 exonuclease activity depends on many parameters, including the reaction conditions. The authors who were first to isolate APE1 from HeLa cells reported that endonuclease enzymatic activity is exhibited within the temperature range of 30-42°C and pH range of 6.7-9.0, while the maximum efficiency was observed at pH 7.5-7.8 [20]. It was recently found [11] that APE1 is involved in the nucleotide incision repair (NIR) pathway, which repairs oxidative DNA damage and removes base modifications (such as 5,6-dihydrodeoxyuridine, 5,6-dihydrothymidine, 5-hydroxydeoxyuridine, α-deoxyadenosine, and α-thymidine) without DNA glycosylases. It was mentioned that this activity is displayed under conditions similar to the optimum of 3′-5′ exonuclease activity (Table 4).

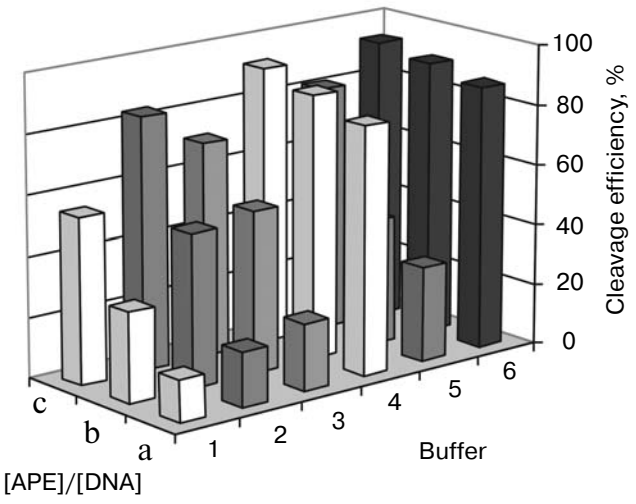


Fig. 4. APE1 3′-5′ exonuclease activity in different buffers towards DNA duplex containing FABGdCMP/dAMP at the 3′ end of the nick. a, b, and c stand for 1, 10, and 100 (for buffers 1 and 2) and 0.25, 1, and 4 (for buffers 3-6), respectively.

We have determined the efficiency of APE1 3′-5′ exonuclease activity towards the residues of native nucleotide and photoreactive analogs at the 3′ end of the primer under the following conditions: (i) under conditions of DNA polymerase and DNA ligase reactions (buffer 1, Table 2), and (ii) under conditions optimal for NIR activity [11] (buffer 6, Table 2). It was found that dNMP cleavage efficiency for all types of DNA duplexes was significantly higher in buffer 6 compared to buffer 1. Since these two buffers result in conditions with different parameters, we varied these parameters step by step using four intermediate buffers for this purpose (Table 2). The efficiency of 3′-5′ exonuclease cleavage of native dNMP (Fig. 4 and Table 3) gradually increased for all investigated analogs (except for FABOdCMP) in the following order: $1 > 2 > 3 \geq 5 > 4 > 6$.

DISCUSSION

The interest in exonuclease activity of APE1 increased after a publication of Chou et al. [5]. In this work, the enzyme concentration range was similar to the conditions for endonuclease hydrolysis reaction by APE1, which suggests a significant contribution of endonuclease activity of APE1 to error correction in the

process of DNA repair. However, in the later investigations of APE1 exonuclease activity, also including those by the same authors, enzyme concentrations required for display of this activity were higher [3, 8, 10]. It should be noted that effective concentrations of repair proteins at the DNA lesion site are unknown. It is possible that *in vitro* excess of enzymes towards the substrate in the reaction mixture mostly reflects their local concentration at the repair site [21]. Furthermore, it is known that the amount of the repair proteins in various mammalian cells varies within a wide range, for instance, the amount of APE1 varies from 350,000 to 7,000,000 molecules per cell [22]. Consequently, we have used an excess of APE1 towards the DNA substrate, all the more so that exonuclease activity is not detected (or is very weak) at low APE1 concentrations, which prevents monitoring the change in activity under various reaction conditions. It was demonstrated [5] that in the case of native dNMP residues at the 3′ ends of oligonucleotides, APE1 exhibits significant 3′-5′ exonuclease activity, where the rate of removal of mismatched nucleotide is 50-160 times higher (depending on nucleotide pair) than that of matched pair: TG ≥ GG ≥ AG >> CG. In contrast, other researchers [8] did not find dramatic difference in efficiency of nucleotide cleavage at the 3′ end by APE1 depending on "canonicity" of the pair and type of lesion in the DNA duplex (e.g., gap or nick). Nevertheless, the efficiency of nucleotide removal from the TG pair is also the highest: TG >> AG ~ CG. In addition, it was noted that the efficiency of the exonuclease reaction is lower than the efficiency of endonuclease reaction by six orders of magnitude [8]. The data obtained by us demonstrate the following pattern of efficiency of 3′-5′ exonuclease cleavage: CA > TG > TA ≥ CG. This pattern is difficult to compare with the literature data, because such a combination of substrate structures is different from what was studied before; however, the observed tendencies confirm that dNMP residues are more efficiently cleaved from mismatched than from matched pairs.

Efficiency of cleavage of native dNMP by APE1 was in general lower than that of photoreactive dNMP analogs. The FABOdCMP residue was the most prone to removal, whereas FAPdCMP was less prone. As a consequence, FAPdCTP is more preferable for photoaffinity modification of proteins in the reconstructed systems and cell/nuclear extracts in the presence of APE1, and FABGdCTP and FABCdCTP are less preferable, whereas FABOdCTP is not recommended at all. All analogs used by us as substrates for 3′-5′ exonuclease activity of APE1 mimic dCMP and dTMP to different extents. Thus, photoreactive analogs used by us can be applied for modeling of matched and mismatched dNMP for the investigation of the base excision repair pathway.

The role of 3′- and 5′-terminal oligonucleotide structures flanking single strand break or gap has been investigated by different authors. The presence of phosphate at the 3′ end of a single nucleotide gap or break did not influence the efficiency of exonuclease reaction compared to the hydroxyl group at this position [9]. In contrast, the nature of 5′ terminal group had a significant influence on the efficiency of exonuclease activity of APE1 [8-10]; however, in this case the data from different authors are contradictory. In a majority of investigations it was shown that the presence of tetrahydrofuran phosphate at the 5′ end of the oligonucleotide flanking the gap or break decreased the efficiency of dNMP residue removal from the 3′ end of the oligonucleotide compared to DNA containing phosphate group at this position [3, 8, 10], but according to other reports [9] the efficiency was increased. The presence of the phosphate group in the tetrahydrofuran moiety stimulated the removal of nucleotide from the 3′ end [10]. However, the sugar without phosphate (according to [10]), in turn, facilitates this reaction to an even larger extent than the single nucleotide overhang, imitating the "tail" (overhanging ssDNA) formed during the long-patch repair pathway [23]. All authors mention the highest efficiency of exonuclease reaction in the case of 5′ hydroxyl group in the gap or break compared to phosphate or tetrahydrofuran phosphate. For example, phosphate group decreases the efficiency of nucleotide removal 10-fold [9] or 8-fold [10], and tetrahydrofuran phosphate decreases the efficiency 5-fold [9] compared to 5′ hydroxyl group. The results obtained by us are in agreement with data on the highest efficiency of APE1 exonuclease activity in the presence of 5′ hydroxyl group and contribute to the common picture implying the following order: hydroxyl (OH) > phosphate (p) > tetrahydrofuran phosphate (pF) groups.

The dependence of endonuclease and exonuclease activity of APE1 on reaction conditions has been reported in the literature before [3, 19]. It was demonstrated [3, 19, 20] that no appreciable APE1 activity can be detected in the absence of Mg^{2+} since these ions are necessary for catalysis [22, 24]. $MgCl_2$ is required for enzymatic activity of APE1 towards the partial DNA duplex in the optimal concentration range 0.1-1.0 mM and towards the DNA with single strand break in the optimal concentration range 2 mM [3]. In the presence of 2 mM $MgCl_2$, exonuclease activity of APE1 was displayed in a narrow range of KCl concentrations (0-60 mM towards the partial DNA duplex with overhanging template and 50-100 mM for DNA with single strand break) and was completely inhibited at concentrations above 100-150 mM. Endonuclease activity was displayed over a wide range of monovalent salt concentrations (up to 300 mM). The maximum of endonuclease activity was observed at 2 mM $MgCl_2$ and 120 mM KCl or 12.5 mM $MgCl_2$ and 20 mM KCl concentrations [3] (Table 4). Authors have assumed that high ionic strength can induce conformational changes in the protein molecule upon its binding to the AP-site in DNA and thus stimulate dissociation of

enzyme–product complex (with cleaved DNA). Table 4 summarizes the literature data on reaction conditions for various APE1 activities.

Significant differences in reaction conditions were shown for the recently discovered nucleotide incision repair (NIR) compared to AP-endonuclease activity [11] (Table 4). Endonuclease activity reached its maximum at pH 7.8-8.2, whereas NIR activity is maximal at pH 6.4-6.8, which is optimal for binding to DNA AP-site [24]. In another work [3], it was shown that endonuclease activity of APE1 is most efficient at pH 7.7-7.9, and exonuclease activity at pH 7.4 (Table 4). Moreover, endonuclease activity of APE1 was significant over a wide range of KCl concentrations (25-200 mM), whereas NIR activity was dramatically decreased at KCl concentrations higher than 50 mM [11].

As shown previously [25], APE1 exhibits endonuclease activity towards AP-site in ssDNA, which was confirmed by Wilson [12]. The efficiency of this reaction strongly depends on the length and sequence of single strand oligonucleotide [12]. As well as the major endonuclease APE1 activity, cleavage of ssDNA depends on the presence of magnesium ions and does not depend on the presence of DNA glycosylases. This activity is influenced by mutations of Asp120 amino acid residue in the enzyme active site [25]. It should be noted that low APE1 affinity towards ssDNA was previously reported [9]. It is interesting that the optimal conditions for cleavage of AP-site in the ssDNA are those most similar to the conditions for exonuclease (rather than to endonuclease) activity of APE1. Optimal conditions for cleavage of ssDNA with AP-site are: 0.5-2 mM $MgCl_2$ and low concentration of monovalent salt ($\leq$50 mM KCl). At the same time endonuclease activity towards DNA duplex with AP-site was displayed most efficiently at 10 mM $MgCl_2$ and 50 mM KCl or 2 mM $MgCl_2$ and 200 mM KCl [12].

Our data also show that 3′-5′ exonuclease activity of APE1 is more profound under conditions of low ionic strength (0.5 mM $MgCl_2$, 25 mM KCl, buffers 3-6; Table 2, Fig. 4) than under the conditions considered to be optimal for endonuclease activity (10 mM $MgCl_2$ and 50 mM KCl, buffers 1 and 2; Table 2, Fig. 4) according to the literature [3, 12]. We have also found that the change in pH or buffer composition from Tris to Hepes increases the efficiency of exonuclease cleavage of 3′-dNMP from single strand break. Addition of BSA did not influence the cleavage efficiency at pH 8.0 (data not shown) and increased the efficiency of the reaction at pH 6.8 (Table 3, Fig. 4). It has been assumed [3, 12] that the difference in optimal conditions for exonuclease and endonuclease activities of APE1 can be due to high ionic strength facilitating changes in protein conformation upon its binding to DNA. Moreover, it can be supposed that less efficient exonuclease cleavage at higher ionic strength results from stabilization of DNA duplex.

Our results on the efficiency of 3′-5′ exonuclease reaction under different conditions allow determining the optima of this APE1 activity. Since photoreactive and other substituted dNTP analogs are widely used for the investigation of excision repair DNA complexes, also including studies with cell and nuclear extracts, the choice of optimal parameters providing stability of DNA structures containing such modified dNMP is an important task. Our data allow determining suitable reaction conditions for the use of photoreactive dNMP analogs incorporated at the 3′ end of DNA nick for photoaffinity modification of proteins, i.e., for the case when photoreactive residues will not be removed by APE1. FAPdCTP is preferable for photoaffinity modification in APE1-containing systems, whereas FABOdCTP is not recommended for use. Our results confirm the literature data regarding the difference between optimal conditions for different APE1 activities; however, the ranges of optimal conditions partially overlap. It would be interesting to study the question of how this multifunctional enzyme facilitates various reactions in a living cell.

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