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Heavy Metal Ions Affect the Activity of DNA Glycosylases of the Fpg Family

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Abstract—Prokaryotic enzymes formamidopyrimidine-DNA glycosylase (Fpg) and endonuclease VIII (Nei) and their eukaryotic homologs NEIL1, NEIL2, and NEIL3 define the Fpg family of DNA glycosylases, which initiate the process of repair of oxidized DNA bases. The repair of oxidative DNA lesions is known to be impaired *in vivo* in the presence of ions of some heavy metals. We have studied the effect of salts of several alkaline earth and transition metals on the activity of Fpg-family DNA glycosylases in the reaction of excision of 5,6-dihydrouracil, a typical DNA oxidation product. The reaction catalyzed by NEIL1 was characterized by values K_m = 150 nM and k_{cat} = 1.2 min^{-1}, which were in the range of these constants for excision of other damaged bases by this enzyme. NEIL1 was inhibited by Al^{3+}, Ni^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{2+} in Tris-HCl buffer and by Cd^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+} in potassium phosphate buffer. Fpg and Nei, the prokaryotic homologs of NEIL1, were inhibited by the same metal ions as NEIL1. The values of I_{50} for NEIL1 inhibition were 7 μM for Cd^{2+}, 16 μM for Zn^{2+}, and 400 μM for Cu^{2+}. The inhibition of NEIL1 by Cd^{2+}, Zn^{2+}, and Cu^{2+} was at least partly due to the formation of metal–DNA complexes. In the case of Cd^{2+} and Cu^{2+}, which preferentially bind to DNA bases rather than phosphates, the presence of metal ions caused the enzyme to lose the ability for preferential binding to damaged DNA. Therefore, the inhibition of NEIL1 activity in removal of oxidative lesions by heavy metal ions may be a reason for their co-mutagenicity under oxidative stress.

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Key words: oxidative stress, DNA repair, DNA glycosylases, heavy metals

DNA, the major genetic information carrier, can be damaged by various genotoxic factors, which finally results in mutations, carcinogenesis, and aging [1, 2]. DNA-damaging factors are ionizing radiation, UV radiation, incorporation of noncomplementary deoxynucleotides by DNA polymerases, and reactive oxygen species generated during oxidative metabolism [3, 4]. Compounds containing heavy metals are among the most widespread genotoxic agents [5, 6]. Besides the direct damage to DNA by free radicals, the formation of which is catalyzed by transition metal ions, genotoxicity of heavy metals is to a large degree determined by their inhibition of DNA repair [6].

No less than six enzymatic types of DNA repair are known to date: direct reversal, base excision repair, nucleotide excision repair, mismatch repair, recombination repair, and non-homologous end joining [7]. The mechanisms of inhibition of DNA repair by heavy metal compounds have been little studied. Cases of co-mutagenic activity of heavy metal ions have been described previously: the synergic effect of these ions and reactive oxygen species results in an abrupt increase in the level of mutation [8, 9]. Heavy metal ions have been shown to inhibit *in vitro* the XPA protein of the nucleotide excision repair system and some DNA glycosylases – the central enzymes of base excision repair [10-12].

Living organisms usually contain several DNA glycosylases responsible for removal of different types of damaged bases (8 and 11 DNA glycosylases are known for *Escherichia coli* and *Homo sapiens*, respectively) [13, 14]. In accordance with the presence of certain DNA-binding

Abbreviations: AP, apurine-apyrimidine; DHU, 5,6-dihydrouracil; ODN, oligodeoxyribonucleotide; THF, (3-hydroxytetrahydrofuran-2-yl)methyl phosphate.

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motifs in the structure of DNA glycosylases, three major families of these enzymes are distinguished: uracil-DNA glycosylase, endonuclease III (Nth), and formamidopyrimidine-DNA glycosylase (Fpg) [13, 15]. The latter family includes prokaryotic enzymes Fpg and endonuclease VIII (Nei) and their homologs from the higher eucaryotes—NEIL1, NEIL2, and NEIL3 [16]. All these enzymes remove oxidized and reduced nucleobases from DNA; in mammals, inactivation of NEIL1 leads to an increase in cell sensitivity to ionizing radiation [17] and development of metabolic syndrome at the level of the organism [18].

Among all human DNA glycosylases, inhibition by heavy metals has been studied only in 8-oxoguanine-DNA glycosylase (OGG1) [11]. It has been shown for glycosylases of the Fpg family that the prokaryotic enzyme Fpg is inhibited by a number of heavy metals *in vitro*, most probably due to competition for the Zn^{2+} binding center in the "zinc finger" motif in the enzyme molecule [10, 12, 19]. The Nei, NEIL2, and NEIL3 proteins also have a zinc finger, but there is no data on their interaction with heavy metal ions in the literature. However, the zinc finger motif is absent from the sequence of eukaryotic protein NEIL1, which contains no Zn atoms according to X-ray structure analysis [16, 20]. Instead, the NEIL1 protein contains a so-called zincless finger: a β-hairpin carrying no residues capable of binding Zn^{2+}, but very similar in conformation to the zinc fingers of other proteins of the Fpg family. Therefore, comparative analysis of inhibition of DNA glycosylases from the Fpg family by heavy metals is interesting for understanding the mechanism of genotoxicity of heavy metals and their role as co-mutagens under oxidative stress.

The goal of this work was to study the effect of heavy metal ions on the process of base excision repair by eukaryotic DNA glycosylase NEIL1 and the homologous prokaryotic DNA glycosylases Fpg and Nei.

MATERIALS AND METHODS

Oligodeoxyribonucleotides and enzymes. The activities of enzymes Fpg, Nei, and NEIL1 were determined using oligodeoxyribonucleotides DHU23, THF23, C23, and comp23 with the sequences 5′-d(CTCTCCCTTCX-CTCCTTTCCTCT)-3′ where X = DHU (5,6-dihydrouracil), 5′-d(CTCTCCCTTCXCTCCTTTCCTCT)-3′ where X = THF ((3-hydroxytetrahydrofuran-2-yl)methyl phosphate), 5′-d(CTCTCCCTTCCCTC-CTTTCCTCT)-3′, and 5′-d(AGAGGAAAGGAGGG-AAGGGAGAG)-3′, respectively.

Oligodeoxyribonucleotides (ODN) were synthesized by the phosphoramidite method in an automatic solid-phase synthesizer, model 394 (Applied Biosystems, USA), and purified by reversed-phase high-pressure liquid chromatography in a PRP-1 column (Hamilton, USA). Double-stranded ODN substrates were obtained by annealing equimolar amounts of ^{32}P-labeled chains of DHU23, THF23, or C23 with unlabeled complementary comp23 chain. As a result, an undamaged C:G nucleotide pair or its damaged derivatives DHU:G and THF:G were formed. The radioactive label was introduced into ODN at the 5′-end using the polynucleotide kinase of phage T4 (New England Biolabs, USA) and γ-[^{32}P]ATP (Radioizotop, Russia) by a procedure described previously [21]. Fpg, Nei, and NEIL1 were isolated from superproducing *E. coli* strains by schemes described previously [17, 22, 23].

Measurement of enzyme activities. The K_m and V_{max} values of the reaction of DHU excision by NEIL1 were measured in a reaction mixture (20 μl) containing 20 mM potassium-phosphate buffer (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.125 mg/ml BSA, and the labeled ODN substrate DHU23/comp23 in the concentration of 5-1000 nM. The reaction was started by adding the enzyme up to 10 nM, carried out at 37°C for 4 min, and stopped by adding a dye (solution of 0.25% xylene cyanole, 0.25% bromophenol blue, and 20 mM EDTA in deionized formamide) and heating at 95°C for 1 min. Reaction products were separated by electrophoresis in 20% polyacrylamide gel in the presence of 7.2 M urea. Radioactivity of the bands was detected by phosphorimaging of Image Screen K (Kodak, USA) using a Molecular Imager FX instrument (Bio-Rad Laboratories, USA). The kinetic parameters were determined by nonlinear regression using SigmaPlot 8.0 (SPSS Inc., USA).

The Fpg, Nei, and NEIL1 activities in the presence of heavy metal ions were determined in a reaction mixture (20 μl) containing 25 mM potassium-phosphate buffer or 25 mM Tris-HCl buffer (pH 7.5), 50 mM KCl, 0.125 mg/ml BSA, labeled ODN substrate DHU23/comp23 at concentration of 50 nM, and the following metal salts—$AlCl_3$, $FeSO_4$, $Cu(NO_3)_2$, $MnCl_2$, $Pb(NO_3)_2$, $NiSO_4$, $CuCl_2$, $CoCl_2$, $Cd(NO_3)_2$, $CaCl_2$, $ZnSO_4$ (Sigma-Aldrich, USA)—in the standard concentration (5 mM) or varying concentrations (0-5 mM). The reaction was started by adding the enzyme to the final concentration of 5 nM (Nei and NEIL1) or 2 nM (Fpg), carried out at 37°C for 5 min, and then stopped for the analysis of products as described above. If necessary, the reaction was stopped by adding NaOH to the concentration of 0.1 M, heated for 2 min at 95°C, and neutralized with an equimolar amount of hydrochloric acid.

The binding of metal ions to NEIL1 and DNA substrate was studied in a reaction mixture (20 μl) containing 25 mM potassium-phosphate buffer or 25 mM Tris-HCl buffer (pH 7.5), 50 mM KCl, 5 mM EDTA, 0.125 mg/ml BSA, labeled ODN substrate DHU23/comp23 (50 nM), and metal salts (1 mM). The final concentration of NEIL1 in the reaction mixture was 5 nM. The binding of metal ions to NEIL1 was studied as follows: the enzyme

was incubated in the reaction mixture without the substrate and EDTA for 5 min on ice; metal ions were chelated by adding EDTA to 5 mM and incubated for 2 min more; and the reaction was started by adding the substrate. To study the binding of metal ions with DNA, the substrate was incubated under the above conditions, and then the reaction was started by adding the enzyme. The residual activity of NEIL1 was determined as described above. In all cases the quantitative parameters were estimated by the results of 3-5 independent experiments.

Analysis of NEIL1 binding to DNA. The effect of heavy metal ions on the formation of NEIL1–DNA complexes was studied by the electrophoretic mobility shift assay. A standard reaction mixture contained 1 nM uncleavable DNA ligand THF23/comp23 or C23/comp23, 25 mM Tris-HCl (pH 7.5), 50 mM KCl, 0 or 1 mM metal salts, 0.125 mg/ml BSA, 10% (v/v) glycerol, and 400 nM NEIL1. The mixture was incubated for 5 min on ice and then loaded on an 8% polyacrylamide gel with the voltage (300 V) already applied. Electrophoresis was performed under nondenaturing conditions (without urea, in 0.5× Tris-borate buffer, pH 8.3, 8°C, 200 V). Radioactivity of the bands was determined by phosphorimaging of Image Screen K using the Molecular Imager FX.

RESULTS

Activities of Fpg DNA glycosylases in the presence of heavy metal salts. A double-stranded ODN with the base DHU paired with G was used as a substrate to measure the kinetic parameters and determine the effect of heavy metal ions on the activity of DNA glycosylases of the Fpg family. DHU is formed in DNA from cytosine under the action of ˙OH radicals under ionizing radiation (~1 DHU residue per 2500 cytosines [24]). This base is mutagenic, since DNA polymerases quite efficiently incorporate dAMP opposite dihydropyrimidines, which potentially results in C→T transition [25, 26]. An important advantage of DHU over other oxidized pyrimidine bases in the model substrate is the possibility of its introduction into ODN during phosphoramidite DNA synthesis. DHU is excised from DNA by a number of DNA glycosylases including Fpg and Nei [27, 28]. There are no data on DHU cleavage by NEIL1 in the literature; however, taking into consideration the homology of NEIL1, Fpg, and Nei, one may expect that DHU will be a substrate for NEIL1 as well. The study of the steady-state kinetics of the excision reaction for DHU substrate has shown that $K_m = 150 \pm 20$ nM and $k_{cat} = 1.2 \pm 0.1$ min^{-1} (Fig. 1). Comparison of these data with the K_m and k_{cat} values for NEIL1 excision from DNA substrates containing other damaged bases [17, 29] leads to the conclusion that DHU serves as no less effective substrate for NEIL1 than DNA with other oxidized nitrogen bases.

During the DHU substrate cleavage in the presence of metal salts with Tris-HCl as a buffer, NEIL1 activity noticeably decreased for Al^{3+}, Ni^{2+}, Pb^{2+}, Co^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+}, but was practically unchanged for Mg^{2+}, Mn^{2+}, and Ca^{2+} (Fig. 2a). Potassium-phosphate buffer more exactly reflects the conditions of the intracellular environment and influences the activities of some DNA glycosylases [30, 31]. During the reaction in potassium-phosphate buffer, the enzyme activity was practically unchanged in the absence of metal ions or in the presence of Mg^{2+}, decreased 1.9-3.0 times for Fe^{2+}, Mn^{2+}, and Ca^{2+}, and increased 1.4-20.3 times for Al^{3+}, Ni^{2+}, Pb^{2+}, Co^{2+}, Cd^{2+}, Zn^{2+}, and Cu^{2+} (Fig. 2, b and c) as compared with the activity in Tris-HCl buffer. In both buffers used, the enzyme activity in the presence of Cd^{2+}, Zn^{2+}, and Cu^{2+} was noticeably lower than in the absence of metal ions. Partial restoration of NEIL1 activity in potassium-phosphate buffer might be evidence of the competitive binding of Al^{3+}, Ni^{2+}, Pb^{2+}, and Co^{2+} to phosphate anions, while the observed decrease in the enzyme activity in potassium-phosphate buffer in the presence of Mn^{2+} and Ca^{2+} may be associated with the formation of insoluble phosphates of these metals and adsorption of the enzyme on their particles.

The effects of metal salts on the enzyme activities of Fpg and Nei, two prokaryotic homologs of NEIL1, were investigated for comparison. It was found that DHU substrate degradation by these enzymes is inhibited by the same metals as in case of NEIL1, both in Tris-HCl buffer (Fig. 3) and in potassium-phosphate buffer (data not shown). Previously it has been reported that Fpg activity is inhibited by Cd^{2+}, Cu^{2+}, and Hg^{2+} in phosphate buffer

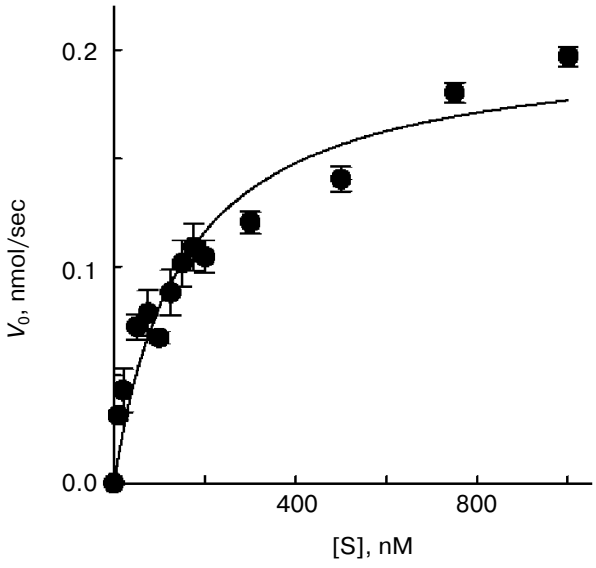

Fig. 1. Kinetics of DHU substrate cleavage by NEIL1. Mean values and standard deviations for four independent experiments are given.

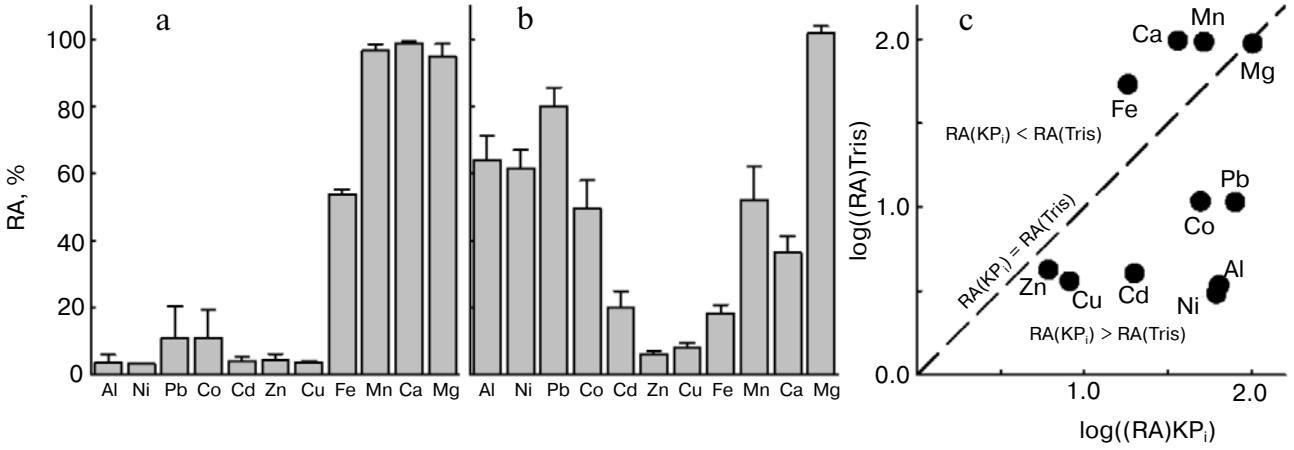

Fig. 2. Effect of metal ions on DHU substrate cleavage catalyzed by NEIL1. a, b) Relative activities (mean and standard deviation in five independent experiments) using potassium-phosphate buffer and Tris-HCl buffer, respectively; c) relative activities (RA, %) in the two buffers.

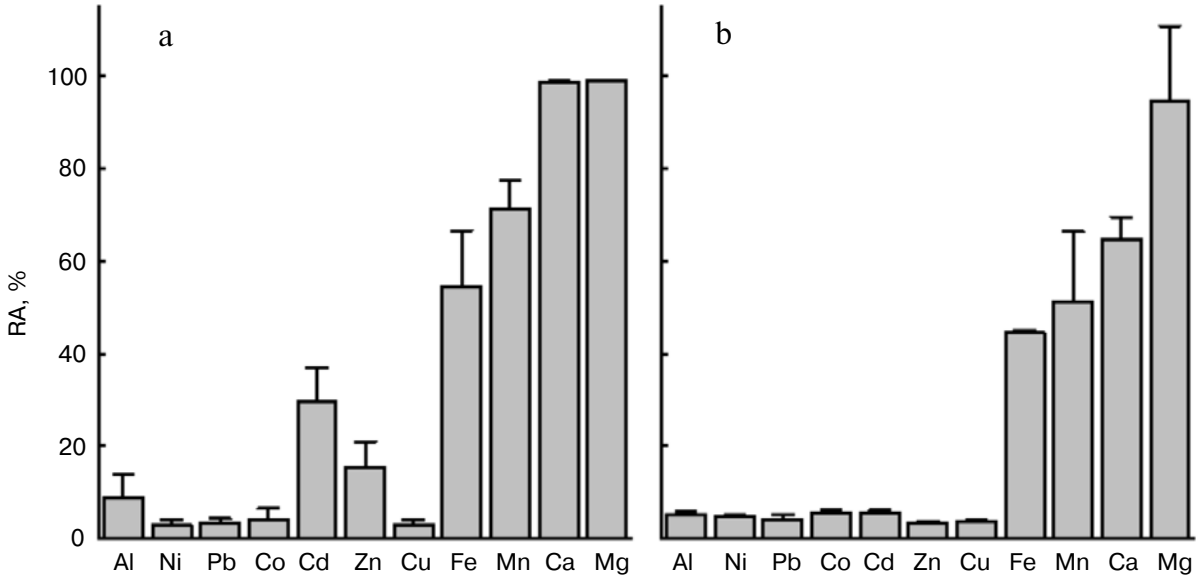

Fig. 3. Relative activities of enzymes Fpg (a) and Nei (b) in DHU substrate cleavage in the presence of metal ions in Tris-HCl buffer.

but not by Pb^{2+}, Co^{2+}, and Ni^{2+} under the same conditions [10, 32], which is in agreement with the data of the present work.

Inhibition of Fpg activity by the above ions might be associated with the fact that they can easily substitute for the Zn atom in the zinc finger structure, which is necessary for the enzyme activity [19, 23]. A zinc finger is also a component of Nei structure, important for its activity [28, 33, 34]; however, this structural motif is absent in NEIL1 on the level of both primary [35] and tertiary structures [20]. PDBSiteScan [36] was used to search for a possible binding center for divalent cations in the structure of NEIL1, but none could be found. Consequently, the inhibition of NEIL1 activity by metal ions might be different in the mechanism from the inhibition of Fpg and Nei enzyme activities, in spite of the homology of sequences and general similarity of spatial structures.

Cd^{2+}, Zn^{2+}, and Cu^{2+} were selected for further study because they inhibited the reaction in both buffers. The I_{50} values at which the enzyme activity was observed to decrease by 50%, were measured (Fig. 4) for Cd^{2+}, Zn^{2+}, and Cu^{2+}: $I_{50} = 7 \pm 2$, 16 ± 7, and 400 ± 95 μM, respectively.

Mechanism of NEIL1 inhibition by heavy metal salts. Enzyme activity might decrease in the presence of metal ions due to their binding to the enzyme, the substrate, or the enzyme–substrate complex, with possible variation of efficiency of different stages of the catalytic reaction.

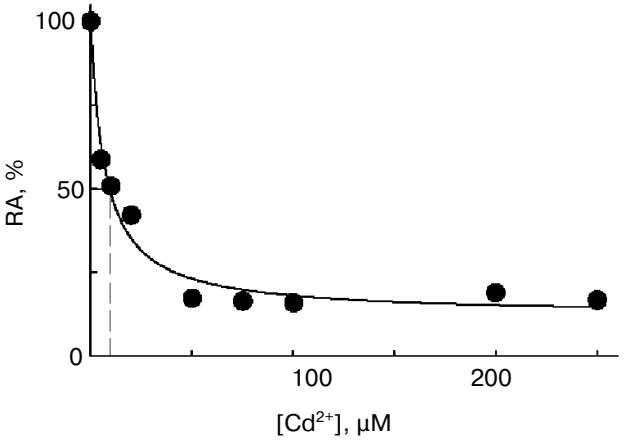

Fig. 4. Dependence of relative activity of NEIL1 in DHU substrate cleaving reaction on Cd^{2+} concentration.

Hence, the effects of Cd^{2+}, Zn^{2+}, and Cu^{2+} at different stages of NEIL1-catalyzed reaction were studied.

Fpg enzymes catalyze several reactions resulting in the removal of a damaged component from DNA. First, these enzymes hydrolyze the *N*-glycoside bond of a damaged deoxynucleoside (DNA glycosylase reaction) with the formation of an alkali-labile apurinic/apyrimidinic site (AP site). This is followed by sequential elimination of phosphate groups from the 3′- and 5′-side of the formed AP site (AP-lyase reaction). As a result, a mononucleoside gap flanked with phosphate groups is formed in the place of the damaged component. When the products of the NEIL1-catalyzed reaction were treated with alkali, the yield of cleaved ODN did not increase (Fig. 5). This is evidence that Cd^{2+}, Zn^{2+}, and Cu^{2+} have no effect on the AP-lyase stage of the reaction but inhibit the reaction already at the DNA-glycosylase stage.

Metal ions were chelated with EDTA to determine whether the inhibiting ions are bound to the protein or to DNA. For this purpose, the enzyme or the substrate was incubated with a metal, then EDTA was added for chelation of free ions, and the reaction was initiated by addition of the substrate or the enzyme, respectively. On chelation of metal ions during preincubation with the enzyme, the activity of the latter was restored almost completely (Fig. 6). On incubation with the DHU substrate, the enzyme was reactivated partially but without complete restoration of activity (Fig. 6). Thus, it is most likely that Cd^{2+}, Zn^{2+}, and Cu^{2+} inhibit NEIL1 due to tight binding to the DNA substrate.

According to X-ray structure analysis, Zn^{2+} in B-DNA is bound only to the peripheral atoms of phosphate groups and also can bind to atom N7 of guanine only in case of significant deviation of DNA from canonic structure [37, 38]. There are no data in the literature on atomic resolution structure for the Cu^{2+} and Cd^{2+} complexes with B-DNA; however, spectroscopic and thermodynamic analyses show that Cd^{2+} and particularly Cu^{2+} are mainly bound not to phosphate groups but to nucleophilic atoms of the nitrogen of heterocyclic bases [39-41]. Consequently, it is probable that conformational and thermodynamic characteristics of the interaction of Cd^{2+}, Zn^{2+}, and Cu^{2+} with damaged and undamaged DNA will differently change their ability to bind to proteins. Therefore, the effects of Cd^{2+}, Zn^{2+}, and Cu^{2+} on the binding of NEIL1 with undamaged and damaged DNA were studied. Double-stranded ODN without modified residues (undamaged DNA) or containing a THF residue,

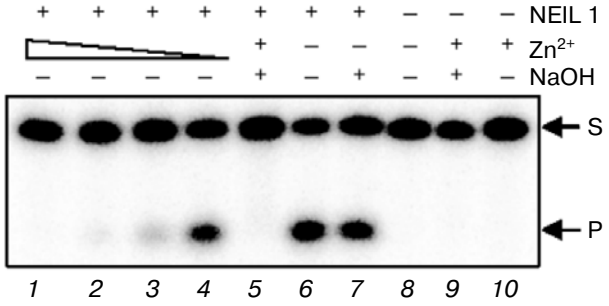

Fig. 5. Products of DHU substrate cleavage by NEIL1 in the presence of different Zn^{2+} concentrations before and after treatment with alkali. Zn^{2+} concentrations (μM): *1*, *5*, *9*, *10*) 5000; *2*) 500; *3*) 50; *4*) 5; *6-8*) 0. Arrows designate substrate (S) and reaction product (P).

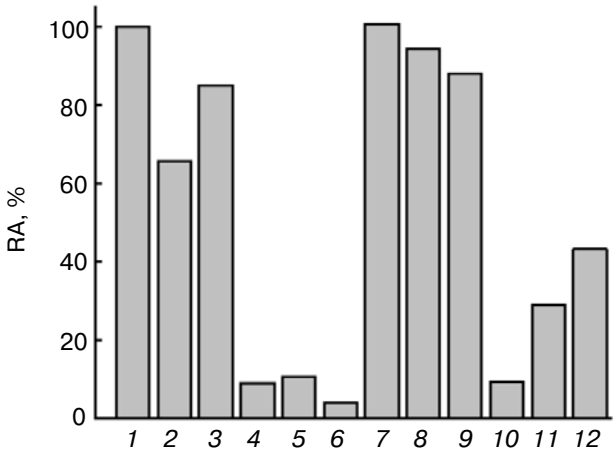

Fig. 6. Relative activity of NEIL1 in DHU substrate cleavage. *1*) Control (reaction in absence of metals and EDTA); *2*) preincubation of enzyme with EDTA followed by substrate addition; *3*) preincubation of substrate with EDTA followed by enzyme addition; *4-6*) reaction in presence of Zn^{2+}, Cd^{2+}, or Cu^{2+}, respectively; *7-9*) preincubation of enzyme with Zn^{2+}, Cd^{2+}, or Cu^{2+}, respectively, followed by EDTA chelation and addition of substrate; *10-12*) preincubation of substrate with Zn^{2+}, Cd^{2+}, or Cu^{2+}, respectively, followed by EDTA chelation and addition of enzyme.

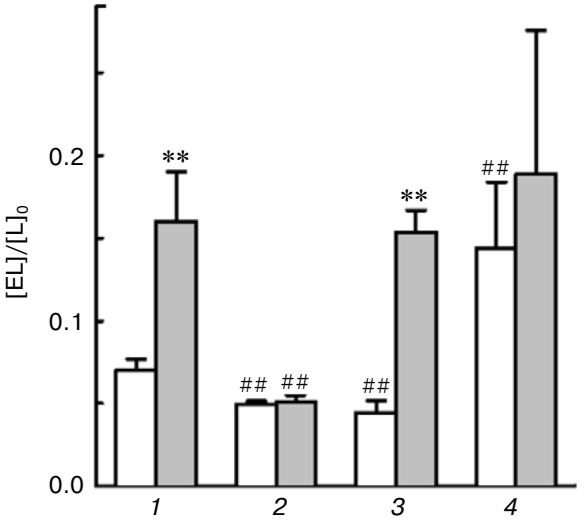

Fig. 7. NEIL1 binding to damaged and undamaged DNA. The fraction of undamaged double-stranded ODN ligand (light columns) or double-stranded ODN ligand containing uncleavable THF residue (dark columns) bound to the enzyme under standard conditions are presented. [EL], concentration of NEIL1–ODN complex; $[L]_0$, total ODN concentration. The binding is shown in the absence of metal ions (*1*) and in the presence of Cd^{2+} (*2*), Zn^{2+} (*3*), and Cu^{2+} (*4*). The mean values and standard deviations of 3-8 independent experiments are given; **, #, and ## designate statistically significant differences according to Student's t-test (#, $p < 0.05$; ** and ##, $p < 0.01$) from the binding of undamaged ODN (**) or from the binding of ODN in the absence of metal ions (#, ##).

i.e. the uncleavable analog of an AP site (damaged DNA), were used as model DNA ligands. In the absence of metal ions, NEIL1 bound the damaged ligand better than the undamaged one (Fig. 7), which was in agreement with the higher affinity of all known DNA glycosylases for damaged DNA. Cd^{2+} reduced the effectiveness of enzyme binding to both damaged and undamaged ODN, while the addition of Zn^{2+} to the reaction mixture reduced only the binding of undamaged DNA ligand. On the contrary, NEIL1 bound undamaged DNA better in the presence of Cu^{2+} than in the absence of metal ions, while the binding of THF-containing ligand was unchanged. As a consequence, in the presence of Cd^{2+} and Cu^{2+} there was no difference between the efficiency of binding of damaged and undamaged DNA by NEIL1, while in the presence of Zn^{2+} the enzyme maintained higher specificity of the binding to damaged DNA (Fig. 7).

DISCUSSION

Previously it has been shown that Cd^{2+} inactivates human OGG1 in two ways: through the binding of metal ion to enzyme molecules and to enzyme–substrate complex [11]. Unlike OGG1, NEIL1 did not bind metal ions and the NEIL1 activity was inhibited mainly due to the formation of metal ion/DNA complexes. OGG1 and NEIL1 are from two different families of DNA glycosylases, which bind DNA through different structural motifs. In particular, in the complexes of DNA and Fpg DNA glycosylases, including NEIL1, a highly conservative residue Arg or Lys lies in the major groove of DNA and probably participates in the primary recognition of a damaged base [16]. However, OGG1 does not form critical contacts with DNA in the major groove [42]. Since Cd^{2+} in complex with DNA is coordinated mainly by the N7 atoms of purine bases lying in the major groove [40], it is quite probable that Cd^{2+} binding does not interfere with the normal functioning of OGG1 but prevents damaged base recognition by NEIL1.

Inhibition of NEIL1 and OGG1 by heavy metal ions, in particular Cd^{2+}, might account for their co-mutagenic properties [8, 9]. Normally, many metals in the cell are components of metal/protein complexes; in particular, metallothioneins bind metals toxic for the cell [43]. However, under the action of protein-damaging agents, e.g. under oxidative stress, these ions may be released and inhibit different cell processes including DNA repair. In particular, during oxidative stress the sulfhydryl groups of metallothioneins are oxidized, and these proteins become unable to capture metal ions [44, 45]. The concentration of toxic metals in complex with metallothioneins can reach significant values. The time of Cd(II) half life in the human body is tens of years, and even at permissible concentrations in the environment this metal accumulates in kidney tissues of an adult up to ~40 μg/g [46]. Complete release of such amount from metal/protein complexes corresponds to sub-millimolar concentrations of Cd^{2+}, and even partial oxidation of metallothioneins might result in the release of the amounts of Cd^{2+} and other metals sufficient for affecting the biochemical processes in cells. Thus, simultaneous oxidative DNA damage and inhibition of DNA repair due to removal of heavy metal ions from metal/protein complexes might result in considerable increase in the mutagenic consequences of oxidative stress.

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