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Substrate Specificity and Excision Kinetics of *Escherichia coli* Endonuclease VIII (Nei) for Modified Bases in DNA Damaged by Free Radicals†

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ABSTRACT: Endonuclease VIII (Nei) is one of three enzymes in *Escherichia coli* that are involved in base-excision repair of oxidative damage to DNA. We investigated the substrate specificity and excision kinetics of this DNA glycosylase for bases in DNA that have been damaged by free radicals. Two different DNA substrates were prepared by γ-irradiation of DNA solutions under N_2O or air, such that they contained a multiplicity of modified bases. Although previous studies on the substrate specificity of Nei had demonstrated activity on several pyrimidine derivatives, this present study demonstrates excision of additional pyrimidine derivatives and a purine-derived lesion, 4,6-diamino-5-formamidopyrimidine, from DNA containing multiple modified bases. Excision was dependent on enzyme concentration, incubation time, and substrate concentration, and followed Michaelis−Menten kinetics. The kinetic parameters also depended on the identity of the individual modified base being removed. Substrates and excision kinetics of Nei and a naturally arising mutant form involving Leu-90→Ser (L90S-Nei) were compared to those of *Escherichia coli* endonuclease III (Nth), which had previously been determined under experimental conditions similar to those in this study. This comparison showed that Nei and Nth significantly differ from each other in terms of excision rates, although they have common substrates. The present work extends the substrate specificity of Nei and shows the effect of a single mutation in the *nei* gene on the specificity of Nei.

Cellular DNA damage is a highly deleterious event to all organisms, and the resulting mutations have been implicated in disease and aging (*1*−*4*). Oxidative damage to DNA bases, caused by oxygen-derived species, is particularly mutagenic and constitutes a major category of DNA damage. Sources of these species are both endogenous, such as normal cellular metabolism, and exogenous, such as ionizing radiation and chemical agents. Hence, a variety of DNA repair pathways have developed to combat DNA damage and to maintain genomic integrity (*5*). The base-excision repair (BER)[1] pathway is the predominant means for repair of damaged bases (*6*), and this pathway is conserved throughout all species. BER is initiated by removal of a damaged base by a DNA glycosylase. In *Escherichia coli*, BER of oxidatively modified bases is initiated by one of three DNA glycosylases (*7*). Nth (endonuclease III) has broad substrate specificity and is primarily responsible for removal of modified pyrimidines (*7*), although it also efficiently removes a purine-derived product, 4,6-diamino-5-formamidopyrimidine (FapyAde) (*8*). On the other hand, formamidopyrimidine DNA glycosylase (Fpg) mainly acts on modified purines such as 8-hydroxyguanine (8-OH-Gua) and formamidopyrimidines in DNA (*7*), but it also removes some modified pyrimidines from oligonucleotides containing one single lesion (*9*−*11*). Nei (endonuclease VIII) was the third glycosylase identified that initiates removal of modified bases from DNA (*12*−*14*). Nei was originally discovered because of the observation that Nth-deficient cells (*nth*$^-$ mutants) are not hypersensitive to DNA-damaging agents (*15*). Therefore, it was proposed that an alternate enzyme might have overlapping functionality

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[1] Abbreviations: BER, base-excision repair; Nei, *E. coli* Nei protein (endonuclease VIII); Nth, *E. coli* Nth protein (endonuclease III); Fpg, formamidopyrimidine DNA glycosylase; GC/IDMS, gas chromatography/isotope-dilution mass spectrometry; FapyAde, 4,6-diamino-5-formamidopyrimidine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH-Gua, 7,8-dihyro-8-oxoguanine, 8-hydroxyguanine; 5,6-diOH-Cyt, 5,6-dihydroxycytosine; 5,6-diOH-Ura, 5,6-dihydroxyuracil (isodialuric acid); CytGly, cytosine glycol; UraGly, uracil glycol; 5-OH-Cyt, 5-hydroxycytosine; 5-OH-Ura, 5-hydroxyuracil; 5,6-diHUra, 5,6-dihydrouracil; 5-OH-6-HUra, 5-hydroxy-6-hydrouracil; ThyGly, thymine glycol; 5,6-diHThy, 5,6-dihydrothymine; 5-OH-5-MeHyd, 5-hydroxy-5-methylhydantoin; 5-OH-6-HThy, 5-hydroxy-6-hydrothymine.

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with Nth. Subsequently, Nei was identified and its gene, *nei*, was cloned (*13, 16*). Nei has a molecular mass of 29.7 kDa, and is a bifunctional glycosylase/AP lyase that catalyzes $\beta-\delta$ elimination at the site of the damaged base; Nei efficiently cleaves AP sites and exhibits significant homology to bacterial Fpg proteins (*14*).

Nth and Nei were shown to have overlapping substrate specificities, since both possess activity on free radical-induced products of thymine and cytosine (*13, 14, 16*−*18*). However, these two enzymes have little or no sequence similarity (*14*). Nei exhibits significant activity on the purine-derived product, 8-OH-Gua embedded in duplex oligonucleotides (*19, 20*). A recent study suggested that Nth was also involved in the repair of 8-OH-Gua, but only when it is incorporated opposite Gua (*21*). Nei might serve as a backup for both Nth and Fpg in *E. coli*. This is supported by the mutation frequencies reported for mutator strains. Neither *nth*$^-$ nor *nei*$^-$ single-mutant cells have a strong mutator phenotype, or significantly greater sensitivity to H_2O_2 than wild-type cells. However, *nth*$^-$ *nei*$^-$ double-mutant cells have a high spontaneous mutation frequency and greater H_2O_2 sensitivity (*14, 16*). Additionally, the synergism between Nei and "purine-specific" repair was illustrated by the mutator phenotypes of *nei*$^-$ *mutY*$^-$ *fpg*$^-$ triple-mutant bacterial strains. MutY is a part of the cellular repair system, and removes adenine from 8-OH-Gua·Ade mispairs (*22*). Removing the Nei function from *mutY*$^-$ *fpg*$^-$ cells resulted in an almost 3-fold increase in mutation rate over *mutY*$^-$ *fpg*$^-$ cells. Cells lacking all three glycosylases had a 600-fold increase in mutation frequency over wild-type cells (*19*).

In the past, the substrate specificity and excision kinetics of Nei were investigated using oligonucleotides containing a single modified base embedded at a defined position (*12, 14, 16, 18*−*20*). The objective of the present study was to determine the substrate specificity and excision kinetics of Nei using free radical-damaged DNA, which contained a multiplicity of modified bases from all four DNA bases. Furthermore, we wished to determine the effect on substrate specificity of a naturally arising single amino acid substitution in Nei, with a mutation at position 90 from leucine to serine (L90S-Nei). We used the technique of gas chromatography/isotope-dilution mass spectrometry (GC/IDMS), which permits the simultaneous measurement of the products of all four DNA bases in a given DNA sample. Thus, this technique facilitates the determination of substrate specificities and excision kinetics of DNA glycosylases for a multitude of modified bases in DNA under identical conditions (see, e.g., ref *8*).

EXPERIMENTAL PROCEDURES

Materials.[2] Modified DNA bases, their stable isotope-labeled analogues, and other materials for GC/IDMS were obtained as described (*23, 24*).

Expression and Purification of Recombinant Nei. Expression and purification was performed as previously described (*20*). The *nei* gene in the pET22b vector (Novagen) was transformed into *E. coli* DE884 *nei*$^-$*mutM*$^-$ cells. Cells were grown in 1 L of LB broth containing 5 μM $ZnCl_2$ and 100 μg/mL ampicillin. At an OD_{550} of 0.5, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and the culture was grown overnight at room temperature. Cells were harvested by centrifugation at 8000 rpm for 15 min. Cells were lysed by BugBuster Protein Extraction Reagent (Novagen) as per the manufacturer's instructions. Cell debris was removed by centrifugation, and the supernatant fraction was then treated with 0.1% Polymin P, followed by ammonium sulfate precipitation (60% saturation). The ammonium sulfate pellet was resuspended and then dialyzed into buffer A (20 mM Tris-HCl, pH 7.5, 1 mM DTT, 10% glycerol, 100 mM NaCl) overnight. The dialysate was loaded onto a 5 mL Hitrap-SP column (Amersham Pharmacia Biotech), and the column was washed extensively with buffer A. Nei protein was eluted in a 0.1−0.5 mM NaCl gradient. Fractions were examined for purity by SDS−PAGE. Pure fractions were pooled and dialyzed into storage buffer (buffer A with 50% glycerol), and then stored at −20 °C. Protein concentrations were determined by using BioRad Protein Assay Kit with BSA as the standard. The recombinant Nei was determined to be ≥95% pure by Coomassie staining.

Preparation of DNA Samples, Enzymic Assays, and GC/IDMS. Calf thymus DNA (Sigma) was dissolved at 4 °C in 10 mM phosphate buffer (pH 7.4) at a concentration of 0.3 mg/mL. Aliquots of this solution were bubbled with N_2O or air and γ-irradiated in a ^{60}Co γ-source at a dose of 80 Gy (dose rate 32 Gy/min). Subsequently, irradiated and unirradiated samples were dialyzed against 10 mM phosphate buffer (pH 7.4) for 18 h at 4 °C using dialysis bags with a molecular weight cutoff of 6000−8000. Phosphate buffer outside the dialysis bags was changed 3 times during the course of dialysis. Enzymatic assays were performed as previously described (*8*). Inactivation of the enzyme was performed by heating at 150 °C for 30 min. For the determination of the dependence of excision on the enzyme amount, 0.25, 0.5, 1, 1.5, or 2 μg of Nei or L90S-Nei was used, and the samples were incubated for 30 min at 37 °C. The measurement of time dependence of excision was performed by incubation of the samples with 1 μg of the enzyme at 37 °C for 10, 20, 30, and 45 min. Excision kinetics were measured as described (*8*). For this purpose, DNA samples were incubated for 30 min at 37 °C with 1 μg of enzyme, which corresponded to an enzyme concentration of 300 nM. The analysis by GC/IDMS of supernatant fractions and pellets from enzymic assays was carried out as described (*8*).

RESULTS

Substrates of Nei and L90S-Nei. While sequencing the *E. coli nei* gene from our plasmid construct isolated from a single clone for verification, we found that it contained a spontaneous T→C point mutation, which had not previously been reported. The published wild-type *nei* sequence codes for a leucine at amino acid 90; however, this clone coded for a serine at position 90, and the protein was termed L90S-Nei. Wild-type Nei and this naturally occurring mutant form were expressed and purified from *nei*$^-$ *fpg*$^-$ *E. coli*. This bacterial strain was used to avoid any complications in data

[2] Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

interpretation from background endogenous enzymic activities. The ability of Nei and L90S-Nei proteins to excise modified bases from free radical-damaged DNA was investigated using an experimental design, which specifically attempts to mimic physiological conditions in which the enzyme must locate damaged sites within vast arrays of undamaged DNA. Thus, the relative kinetics of release of modified bases represent a composite of competition with undamaged DNA, relative binding constants of damaged and undamaged DNA, and rates of excision. This is in contrast to previous data obtained using oligonucleotides containing a single lesion at a defined position. For this study, DNA samples were γ-irradiated under N_2O (anoxic) or air (oxic). Using GC/IDMS, 17 modified bases were identified in DNA γ-irradiated under N_2O conditions (*8*). These lesions are produced in DNA by reactions of hydroxyl radical with DNA bases, with the exception of 5,6-dihydrothymine and 5,6-dihydrouracil (a deamination product of 5,6-dihydrocytosine), which result from reactions of radiation-generated H atoms (reviewed in refs *25, 26*). For DNA that was γ-irradiated under air, 13 modified bases were identified and quantified. In the presence of oxygen, 5,6-dihydrothymine (5,6-diHThy), 5,6-dihydrouracil (5,6-diHUra) (a deamination product of 5,6-dihydrocytosine), 5-hydroxy-6-hydrothymine (5-OH-6-HThy), and 5-hydroxy-6-hydrouracil (5-OH-6-HUra) (a deamination product of 5-hydroxy-6-hydrocytosine) are not formed because of the scavenging of H atoms by oxygen and because of diffusion-controlled reactions of 5-hydroxyl adduct radicals of thymine and cytosine with oxygen (*25, 26*).

DNA samples were incubated with either active enzyme or heat-inactivated enzyme, or no enzyme. Supernatant fractions and pellets of DNA samples were separated and analyzed by GC/IDMS. Of the modified bases in irradiated DNA samples, Nei and L90S-Nei efficiently excised FapyAde, thymine glycol (ThyGly), 5-OH-6-HThy, 5-OH-6-HUra, 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd), uracil glycol (UraGly), 5-hydroxyuracil (5-OH-Ura), 5,6-dihydroxycytosine (5,6-diOH-Cyt), 5,6-dihydroxyuracil (5,6-diOH-Ura) (isodialuric acid), 5,6-diHThy, and 5,6-diHUra. Uracil derivatives are formed by the deamination of cytosine derivatives during either irradiation or workup of samples, or both (*25, 26*). The excision of modified bases was assessed by their appearance in supernatant fractions of DNA samples incubated with active enzyme. The amounts of modified bases in pellets from DNA samples incubated with active enzyme were significantly reduced when compared to those in pellets from DNA samples incubated with heat-inactivated enzyme or without any enzyme. In the case of the DNA samples incubated with active enzyme, the excised amounts found in supernatant fractions were similar to those removed from the pellets, indicating the unequivocal excision of modified bases. Other modified bases, 5-hydroxycytsosine (5-OH-Cyt), alloxan, 8-hydroxyadenine, 2-hydroxyadenine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 8-OH-Gua, and 5-(hydroxymethyl)uracil, were not significantly excised. Figure 1 illustrates the structures of the substrates of Nei. For comparison, the structures of 5-OH-Cyt and alloxan, which are the substrates of *E. coli* Nth, are also included in this figure. Excision of five modified bases as a function of the amount of Nei is shown in Figure 2. The absolute amount of excision products increased with

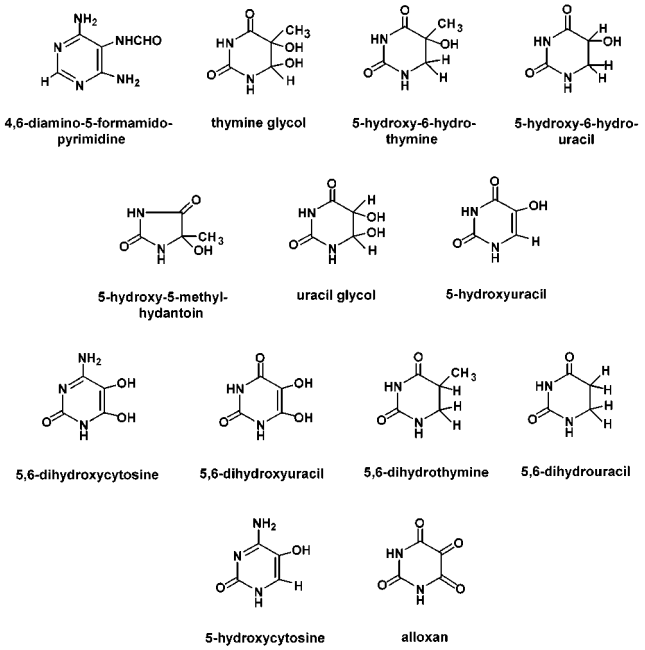

FIGURE 1: Structures of the compounds discussed in this study. Structures of alloxan and 5-OH-Cyt are shown for comparison purposes only. In this study, these compounds were not identified as substrates of Nei or L90S-Nei.

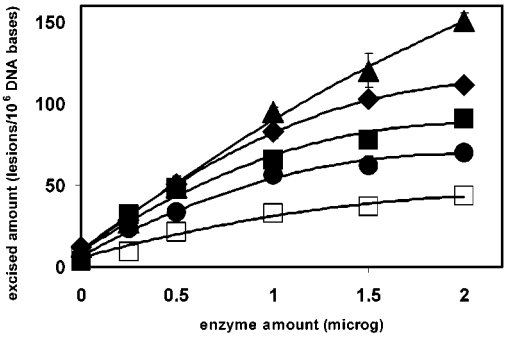

FIGURE 2: Excision of modified bases by Nei as a function of the enzyme amount. DNA γ-irradiated under N_2O (100 μg) was used as a substrate. The incubation time was 30 min at 37 °C. The amounts given on the *y*-axis represent those found in the supernatant fractions. (▲) 5-OH-6-HThy; (◆) FapyAde; (■) 5-OH-6-HUra; (●) 5-OH−Ura; (□) ThyGly.

increasing amounts of Nei from 0.25 to 2 μg and reached a plateau above 2 μg of enzyme. Time dependence of excision was measured using 1 μg of the enzyme and incubation times of 10, 20, 30, and 45 min. Excision increased as a function of time and reached a plateau above 45 min (data not shown).

Excision Kinetics. Kinetic parameters were determined by measurement of excision at six different concentrations of modified bases with the total amount of DNA remaining constant in each sample. Table 1 shows the concentration ranges of modified bases in DNA samples used for kinetic measurements. Excised amounts of modified bases in supernatant fractions were used for these determinations. Kinetic parameters of 5,6-diHThy and 5,6-diHUra could not be determined because of low excision. It should also be pointed out that 5-OH-Cyt, 5-OH-Ura, and UraGly are dehydration and deamination products of cytosine glycol (CytGly) in DNA (*25*−*27*). CytGly was not observed in supernatant fractions of DNA samples incubated with active enzyme; however, *cis*- and *trans*-uracil glycols and 5-OH-

Table 1: Concentration Ranges of Modified Bases in DNA Samples Used for Kinetic Studies

modified base	concentration (μM) range: irradiated under N_2O	concentration (μM) range: irradiated under air
FapyAde	0.37−1.7	0.26−1.3
ThyGly	0.27−0.97	0.75−4.6
UraGly + 5-OH-Ura	0.14−0.66	0.44−3.2
5-OH-5-MeHyd	0.45−1.6	0.27−1.4
5,6-diOH-Cyt + 5,6-diOH-Ura		0.34−1.7
5-OH-6-HThy	0.71−4.7	
5-OH-6-HUra	0.24−1.3	

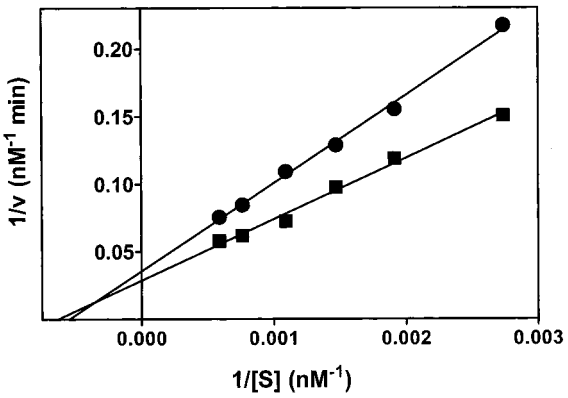

FIGURE 3: Lineweaver−Burk plots for excision of FapyAde by Nei (●) and by L90S-Nei (■) from DNA irradiated under N_2O. The incubation time was 30 min at 37 °C. The enzyme amount was 1 μg/100 μg of DNA. [S], concentration of FapyAde; v, initial velocity of excision of FapyAde. The amounts of products found in supernatant fractions were used for initial velocity.

Ura were detected. It is not known if the enzyme actually excised these modified bases or if they were formed by modification of excised CytGly during workup of the DNA. On the other hand, excision of UraGly, 5-OH-Cyt, and 5-OH-Ura by Nei from oligonucleotides was reported, suggesting that these modified bases are substrates of Nei and would be excised from DNA as well (*14*, *18*). In the present study, kinetic parameters of UraGly and 5-OH-Ura were calculated as a sum, since only 5-OH-Ura was detected in acid hydrolysates of DNA samples. This was also true for 5,6-diOH-Cyt, which deaminates to give 5,6-diOH-Ura upon acidic hydrolysis of DNA (*27*). Both 5,6-diOH-Ura and 5,6-diOH-Cyt were detected in supernatant fractions. Only in the case of DNA irradiated under air were kinetic parameters of these compounds calculated as a sum, because of the low extent of excision from DNA irradiated under N_2O.

Excision of modified bases followed Michaelis−Menten kinetics (*28*). Kinetic constants and standard deviations ($n = 6$) were calculated using a computer program with the linear least-squares analysis of the data. Initial velocities were estimated from plots of products excised as a function of incubation time. Figures 3 and 4 illustrate typical Lineweaver−Burk plots for excision of FapyAde and 5-OH-6-HThy by Nei and L90S-Nei. Kinetic constants determined in this study are given in Tables 2−4, as well as kinetic constants of Nth, which were previously reported (*8*). In the previous study, Nth was prepared from an *E. coli fpg*$^-$ strain and extensively purified by several chromatographic steps (*29*). A comparison of the kinetic constants of *E. coli* Nth with those of human and yeast Nth proteins, and yeast Ntg1

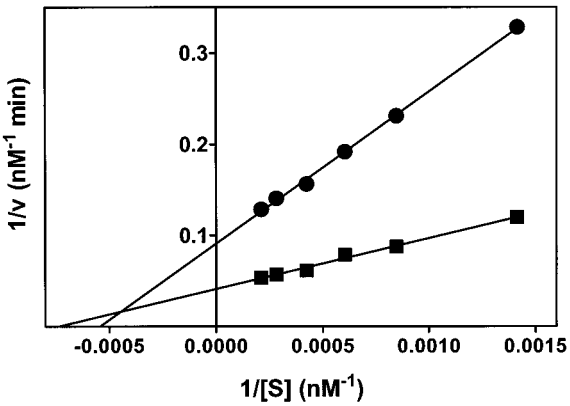

FIGURE 4: Lineweaver−Burk plots for excision of 5-OH-6-HThy by Nei (●) and by L90S-Nei (■) from DNA irradiated under N_2O. The incubation time was 30 min at 37 °C. The enzyme amount was 1 μg/100 μg of DNA. [S], concentration of 5-OH-6-HThy; v, initial velocity of excision of 5-OH-6-HThy. The amounts of products found in supernatant fractions were used for initial velocity.

and Ntg2 proteins, was published elsewhere (*8*). The results of this study show that both Nei and L90S-Nei excised 5-OH-5-MeHyd with the highest maximum velocity (V_{max}) and specificity constant (k_{cat}/K_M) from DNA irradiated under N_2O (Tables 2 and 4), indicating the preference of these enzymes for excision of 5-OH-5-MeHyd. FapyAde and UraGly (total of UraGly and 5-OH-Ura) were the next preferred substrates. The k_{cat}/K_M values for excision 5-OH-6-HThy and 5-OH-6-HUra by L90S-Nei were significantly greater than those by Nei, indicating the greater preference of the former enzyme for these substrates than the latter (Table 4). Otherwise, both enzymes exhibited similar preference for the remaining substrates. In the case of DNA irradiated under air, UraGly was the most preferred substrate by Nei with the greatest k_{cat}/K_M value (Table 4), followed by FapyAde. In contrast, L90S-Nei excised the modified bases with similar preference except for 5,6-diOH-Cyt. For both enzymes, 5,6-diOH-Cyt (total of 5,6-diOH-Cyt and 5,6-diOH-Ura) was the least preferred substrate. Except for UraGly, there was no difference between the preferences of the two enzymes for excision of the products from DNA irradiated under air as indicated by similar k_{cat}/K_M values (Table 4).

DISCUSSION

This study shows the ability of Nei and a naturally occurring mutant form, L90S-Nei, to excise a number of pyrimidine-derived lesions and a purine derivative from DNA damaged by free radicals. Two different DNA substrates were used, which were prepared by γ-irradiation under N_2O or air. They contained a multiplicity of products from all four DNA bases. Previous studies have shown that Nei possesses excision activity for the pyrimidine-derived lesions ThyGly, 5,6-diHThy, 5,6-diHUra, 5-OH-Cyt, 5-OH-Ura, and UraGly from oligonucleotides with a single lesion at a defined position (*12*, *14*, *18*, *20*). Additionally, Nei has been found to also excise the purine-derived 8-OH-Gua paired with Gua, Ade, or Cyt from duplex oligonucleotides (*19*). However, this activity is relatively inefficient and occurs to a much lesser extent than the activity for 5,6-diHUra paired with Ade (*20*). Our present results confirm the excision of ThyGly, 5,6-diHThy, 5,6-diHUra, 5-OH-Ura, and UraGly by Nei. In addition, we demonstrated the excision of other

Table 2: Maximum Velocities [V_{max} (nM min^{-1})[a]] for Excision by Nei, L90S-Nei, and Nth from γ-Irradiated DNA

	irradiation (N_2O)			irradiation (air)		
substrate	Nei	L90S-Nei	Nth	Nei	L90S-Nei	Nth
FapyAde	$28.1 \pm 2.5^{f,g,j,k}$	$35.0 \pm 4.3^{f,g,j,k}$	$28.3 \pm 1.8^{e-h}$	$10.0 \pm 1.0^{b,f,g}$	$5.8 \pm 0.3^{c,d,f,g}$	$8.8 \pm 0.3^{d-g,i}$
ThyGly	$23.5 \pm 11.0^{f,g,j,k}$	$29.0 \pm 9.0^{f,g,k}$	$27.6 \pm 1.7^{f-h,j,k}$	$13.5 \pm 0.3^{c,f,g}$	$12.0 \pm 0.5^{c,f,g,i}$	$41.1 \pm 7.3^{e,f,h,i}$
5-OH-Cyt	–	–	$16.0 \pm 1.6^{g,h}$	–	–	$23.3 \pm 2.4^{g,h}$
UraGly	$3.7 \pm 0.2^{b,c,g,j,k}$	$10.6 \pm 0.8^{g,j,k}$	$14.2 \pm 1.2^{g,h}$	$33.8 \pm 2.3^{b,c,g,i}$	20.5 ± 0.6^{i}	$25.0 \pm 2.2^{g,h}$
5-OH-5-MeHyd	$86.8 \pm 15^{c,j,k}$	$72.2 \pm 8.8^{c,j,k}$	9.2 ± 1.5^{j}	$42.4 \pm 10.8^{b,i}$	$22.7 \pm 5.7^{c,i}$	$41.2 \pm 1.9^{h,i}$
alloxan	–	–	8.9 ± 2.7^{j}	–	–	7.6 ± 0.8^{i}
5,6-diOH-Cyt	–	–	–	$9.3 \pm 1.1^{b,c}$	5.3 ± 0.3^{c}	21.3 ± 1.9
5-OH-6-HThy	$11.0 \pm 0.3^{b,c}$	24.3 ± 1.1^{c}	17.4 ± 0.4^{k}	–	–	–
5-OH-6-HUra	$7.4 \pm 1.1^{b,c}$	18.8 ± 4.3^{c}	10.6 ± 1.1	–	–	–

[a] Values represent the mean ± standard deviation ($n = 6$); values for Nth are from ref *8*. ([Nei] = 300 nM, [Nth] = 757 nM.) [b] Statistically different from the value in column 2 ($P < 0.05$). [c] Statistically different from the value in column 3 ($P < 0.05$). [d] Statistically different from the value in row 2 ($P < 0.05$). [e] Statistically different from the value in row 3 ($P < 0.05$). [f] Statistically different from the value in row 4 ($P < 0.05$). [g] Statistically different from the value in row 5 ($P < 0.05$). [h] Statistically different from the value in row 6 ($P < 0.05$). [i] Statistically different from the value in row 7 ($P < 0.05$). [j] Statistically different from the value in row 8 ($P < 0.05$). [k] Statistically different from the value in row 9 ($P < 0.05$).

Table 3: Michaelis Constants [K_M (nM)[a]] for Excision by Nei, L90S-Nei, and Nth from γ-Irradiated DNA

	irradiation (N_2O)			irradiation (air)		
substrate	Nei	L90S-Nei	Nth	Nei	L90S-Nei	Nth
FapyAde	1828 ± 172^{f}	1586 ± 212^{f}	$1264 \pm 86^{f-h}$	$635 \pm 77^{b,c,d,f,g,i}$	$312 \pm 26^{d,f,g}$	$343 \pm 18^{d-i}$
ThyGly	$3115 \pm 550^{d,f,j,k}$	2371 ± 749^{f}	$1654 \pm 104^{e-k}$	$1109 \pm 73^{c,f}$	$919 \pm 39^{c,g,i}$	$2832 \pm 577^{e,g-i}$
5-OH−Cyt	–	–	863 ± 95^{g}	–	–	$610 \pm 102^{f-i}$
UraGly	$128 \pm 17^{g,j,k}$	$440 \pm 40^{b,g,j,k}$	$513 \pm 51^{j,k}$	$2098 \pm 154^{b,i}$	$785 \pm 32^{c,g}$	2028 ± 202^{g}
5-OH-5-MeHyd	2569 ± 476^{c}	$2112 \pm 270^{c,k}$	$444 \pm 78^{j,k}$	1707 ± 442^{b}	$3051 \pm 789^{c,i}$	1144 ± 57
Alloxan	–	–	624 ± 204	–	–	1375 ± 167
5,6-diOH−Cyt	–	–	–	1010 ± 142^{b}	509 ± 50^{c}	1554 ± 104
5-OH-6-HThy	1838 ± 61^{c}	1351 ± 90	930 ± 30	–	–	–
5-OH-6-HUra	1473 ± 226	1143 ± 279	978 ± 113	–	–	–

[a] Values represent the mean ± standard deviation ($n = 6$); values for Nth are from ref *8*. ([Nei] = 300 nM, [Nth] = 757 nM.) [b] Statistically different from the value in column 2 ($P < 0.05$). [c] Statistically different from the value in column 3 ($P < 0.05$). [d] Statistically different from the value in row 2 ($P < 0.05$). [e] Statistically different from the value in row 3 ($P < 0.05$). [f] Statistically different from the value in row 4 ($P < 0.05$). [g] Statistically different from the value in row 5 ($P < 0.05$). [h] Statistically different from the value in row 6 ($P < 0.05$). [i] Statistically different from the value in row 7 ($P < 0.05$). [j] Statistically different from the value in row 8 ($P < 0.05$). [k] Statistically different from the value in row 9 ($P < 0.05$).

Table 4: Specificity Constants [$k_{cat}/K_M \times 10^5$ (min^{-1} nM^{-1})[a]] for Excision by Nei, L90S-Nei, and Nth from γ-Irradiated DNA

	irradiation (N_2O)			irradiation (air)		
substrate	Nei	L90S-Nei	Nth	Nei	L90S-Nei	Nth
FapyAde	$5.0 \pm 0.3^{c,d,f,g,j,k}$	$7.2 \pm 0.6^{c,d}$	3.1 ± 0.3^{k}	5.2 ± 0.5^{f}	6.1 ± 1.9^{i}	$3.5 \pm 0.2^{f,h}$
ThyGly	$2.5 \pm 0.8^{f,g}$	$4.0 \pm 0.8^{c,f,g}$	2.3 ± 0.2	3.5 ± 1.0^{f}	$4.8 \pm 0.7^{c,}$	$2.0 \pm 0.5^{e,g,h}$
5-OH-Cyt	–	–	2.5 ± 0.4	–	–	$5.2 \pm 0.9^{f,h,i}$
UraGly	$9.5 \pm 0.4^{c,j,k}$	7.9 ± 0.4^{c}	3.8 ± 0.5^{k}	$8.6 \pm 0.2^{c,g,i}$	$5.3 \pm 0.2^{c,i}$	$1.7 \pm 0.2^{g,h}$
5-OH-5-MeHyd	$11.1 \pm 1.3^{c,j,k}$	$11.2 \pm 0.9^{c,j,k}$	2.8 ± 0.7	4.3 ± 0.7	4.5 ± 0.8^{i}	$4.9 \pm 0.3^{h,i}$
alloxan	–	–	1.9 ± 0.9	–	–	0.8 ± 0.1^{i}
5,6-diOH-Cyt	–	–	–	3.4 ± 0.1	3.0 ± 0.2	1.9 ± 0.2
5-OH-6-HThy	2.0 ± 0.1^{b}	5.9 ± 0.2^{c}	2.6 ± 0.1	–	–	–
5-OH-6-HUra	1.6 ± 0.2^{b}	5.4 ± 0.8^{c}	1.5 ± 0.2	–	–	–

[a] Values represent the mean ± standard deviation ($n = 6$); values for Nth are from ref *8*. ($k_{cat} = V_{max}$/[enzyme]; [Nei] = 300 nM, [Nth] = 757 nM.) [b] Statistically different from the value in column 2 ($P < 0.05$). [c] Statistically different from the value in column 3 ($P < 0.05$). [d] Statistically different from the value in row 2 ($P < 0.05$). [e] Statistically different from the value in row 3 ($P < 0.05$). [f] Statistically different from the value in row 4 ($P < 0.05$). [g] Statistically different from the value in row 5 ($P < 0.05$). [h] Statistically different from the value in row 6 ($P < 0.05$). [i] Statistically different from the value in row 7 ($P < 0.05$). [j] Statistically different from the value in row 8 ($P < 0.05$). [k] Statistically different from the value in row 9 ($P < 0.05$).

pyrimidine-derived lesions 5,6-diOH-Ura (isodialuric acid), 5,6-diOH-Cyt, 5-OH-6-HThy, 5-OH-6-HUra, and 5-OH-5-MeHyd, and a purine derivative, FapyAde. However, we did not observe detectable excision of 5-OH-Cyt or 8-OH-Gua from DNA with multiple modified bases in contrast to excision by Nei of these lesions from oligonucleotides containing one single modified base. This study extends the substrate specificity of Nei and reports simultaneously measured excision kinetics of the substrates. The measurement of excision kinetics facilitated the comparison of the properties of Nei with those of other DNA glycosylases such as *E. coli* Nth (this study), human Nth, *Schizosaccharomyces pombe* Nth, and *Saccharomyces cerevisiae* Ntg1 and Ntg2 (*8*).

Nei and Nth have no sequence homology and no similar structural motifs (*13*, *16*). However, in vivo mutation frequencies and in vitro biochemical analyses clearly show that the two enzymes share functional homology. The results

presented in Tables 2−4 show that Nei and Nth of *E. coli* act on similar substrates, but differ in their substrate preferences as shown by the determined specificity constants. Nei exhibited significantly greater specificity than Nth for FapyAde, UraGly, and 5-OH-5-MeHyd for excision from DNA irradiated under N_2O. In the case of DNA irradiated under air, Nei exhibited greater preference than Nth for FapyAde, ThyGly, and UraGly. Both Nei and Nth had similar 5-OH-5-MeHyd activity in DNA irradiated under air; however, Nei showed much higher specificity than Nth for excision of 5-OH-5-MeHyd from DNA irradiated under N_2O. The underlying mechanisms of excision from these two different DNA substrates leading to differences in excision kinetics are not known. Because oxygen inhibits the formation of certain modified bases and because the yields of modified bases significantly differ from one another, the distribution of modified bases along the DNA sequence might be different in these DNA substrates, giving rise to such differences. Therefore, it is plausible that excision kinetics of these enzymes in vivo might also depend on the DNA-damaging agent.

Neither enzyme significantly excised 5,6-diOH-Ura or 5,6-diOH-Cyt from DNA irradiated under N_2O to permit the determination of excision kinetics. However, they excised these compounds with similar preference from DNA irradiated under air. These results clearly show the differences between Nei and Nth in terms of substrates and excision kinetics, and the differences between the rates of excision from two different DNA substrates. The most notable difference in substrate specificity is the excision of alloxan and 5-OH-Cyt. Nei did not excise alloxan in either DNA sample. In contrast, Nth excises this product with a preference similar to that of other substrates (*8*). Similarly, 5-OH-Cyt is among the major substrates of Nth, but was not significantly excised by Nei. We do not know the reason for this difference between Nei and Nth. Since these enzymes possess no sequence homology, it may well be that their active sites are different, and, thus, Nei does not recognize alloxan or 5-OH-Cyt. On the other hand, it is not known whether Nei was not able to excise 5-OH-Cyt from DNA or whether CytGly was completely converted into UraGly and 5-OH-Ura under the conditions of incubation with active enzyme. 5-OH-Cyt is a stable compound and is not expected to deaminate, leading to 5-OH-Ura under the mild conditions of enzymic hydrolysis. This assertion is supported by the fact that 5-OH-Cyt was detected in acid hydrolysates of DNA. However, excision of 5-OH-Cyt by Nei from oligonucleotides has been previously reported (*14*).

Although Nei and Nth have no sequence homology, Nei exhibits strong sequence homology in N- and C-terminal sequences to five bacterial Fpg proteins including *E. coli* Fpg (*13*). This study also demonstrates overlapping functionality between these two enzymes. We have shown that the purine derivative FapyAde, which is a known substrate of Fpg (*29*, *30*), is efficiently excised by Nei. However, these two enzymes have no other common substrates. *E. coli* Fpg excises FapyGua and 8-OH-Gua with similar excision kinetics in addition to FapyAde (*30*), but Nei does not release either FapyGua or 8-OH-Gua from DNA. As mentioned above, this is in contrast to previous reports showing excision of 8-OH-Gua by Nei with low efficiency from oligonucleotides, which had a single 8-OH-Gua molecule at a defined position (*19*, *20*), while the DNA samples in this study contained many modified bases. Thus, the discrepancy might be due to the types of assays and substrates used. Competition for Nei active sites among such a variety of lesions may leave a poor substrate such as 8-OH-Gua unprocessed in favor of lesions with higher enzyme affinity.

A mutation in the *nei* gene of *E. coli* leading to the replacement of Leu-90 by Ser conserved the activity of Nei, although there were some significant differences between the excision kinetics of Nei and L90S-Nei. The mutated form exhibited a greater preference than the wild-type enzyme for excision of 5-OH-6-HThy, 5-OH-6-HUra, FapyAde, and ThyGly from DNA irradiated under N_2O. In the case of DNA irradiated under air, excision rates of both enzymes were similar with the exception of UraGly, for which L90S-Nei exhibited a greater preference than Nei. Hence, this small genetic drift appears to be a tolerable mutation in the Nei protein that has no large effects on enzyme activity. This result is not surprising given what we know about this amino acid position in the Nei-Fpg family. As mentioned previously, Nei and Fpg proteins share significant sequence homology. The amino acid at position 90 is not conserved among this family. Among an alignment of 11 family members, including Nei, this position was occupied by 6 different amino acids. Additionally, the crystal structure of Fpg from *Thermus thermophilus* has recently been reported (*31*). Assuming structural similarity between this protein and Nei, Leu-90 is far removed from the enzyme's active site. We would not expect this amino acid to contribute significantly to enzyme activity or substrate recognition.

In conclusion, we discovered additional substrates of Nei and established its excision kinetics for multiple modified bases in DNA. One of the substrates, FapyAde, is a purine derivative, which is also a substrate of Nth of *E. coli*. The results facilitated a comparison of the substrate specificity and excision kinetics of Nei with those of Nth. These enzymes act on similar substrates, although they exhibit different excision kinetics, no sequence homology, and no similar structural motifs. This study extends the substrate specificity of Nei for a multitude of modified bases in DNA and reports their simultaneously measured excision kinetics for the first time.

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