Novel Substrates of *Escherichia coli* Nth Protein and Its Kinetics for Excision of Modified Bases from DNA Damaged by Free Radicals†

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ABSTRACT: *Escherichia coli* Nth protein (endonuclease III) is a DNA glycosylase with a broad substrate specificity for pyrimidine derivatives. We discovered novel substrates of *E. coli* Nth protein using gas chromatography/isotope-dilution mass spectrometry and DNA samples, which were damaged by γ-irradiation or by H_2O_2/Fe(III)-EDTA/ascorbic acid. These were 4,6-diamino-5-formamidopyrimidine, 5,6-dihydroxyuracil, and 5,6-dihydroxycytosine. The first compound was recognized for the first time as a purine-derived substrate of the enzyme. We also investigated kinetics of excision of a multitude of modified bases from three damaged DNA substrates. Excision of modified bases was determined as a function of enzyme concentration, incubation time, and substrate concentration. Excision followed Michaelis−Menten kinetics. Kinetic parameters were determined for the following modified bases: 4,6-diamino-5-formamidopyrimidine, *cis*- and *trans*-thymine glycols, 5-hydroxycytosine, *cis*- and *trans*-uracil glycols, 5-hydroxyuracil, 5-hydroxy-5-methylhydantoin, alloxan, 5,6-dihydroxycytosine, 5,6-dihydroxyuracil, 5-hydroxy-6-hydrothymine, and 5-hydroxy-6-hydrouracil. The results show that three newly discovered substrates were excised by the enzyme with a preference similar to excision of its known major substrates such as thymine glycol and 5-hydroxycytosine. Excision kinetics significantly depended on the nature of the damaged DNA substrates in agreement with previous results on other DNA glycosylases. Specificity constants (k_{cat}/K_M) of *E. coli* Nth protein were compared to those of its previously investigated functional homologues such as human and *Schizosaccharomyces pombe* Nth proteins and *Saccharomyces cerevisiae* Ntg1 and Ntg2 proteins. This comparison shows that significant differences exist with respect to substrate specificity and kinetic parameters despite extensive structural conservation among the Nth homologues.

DNA *N*-glycosylases are involved in the first step of base-excision repair of DNA damage (reviewed in refs *1* and *2*). DNA base damage that results from reactions of reactive oxygen-derived species including free radicals with DNA bases is thought to be repaired in cells mainly by base-excision repair (reviewed in refs *1* and *2*). In *Escherichia coli*, there are several DNA glycosylases that remove modified bases from damaged DNA (*1*, *2*). Nth and Nei proteins (endonucleases III and VIII, respectively) excise pyrimidine-derived lesions, whereas Fpg protein primarily acts on purine modifications (*1*−*15*) but also excises pyrimidine-derived lesions and a ring fragmentation product of thymine C5-hydrate from oligonucleotides (*1*, *10*, *15*).

E. coli Nth protein (Nth-Eco)[1] is one of the most investigated DNA glycosylases since its isolation by Radman (*3*) (reviewed in refs *1* and *2*). This enzyme with a molecular mass of 24 kDa is encoded by the *nth* gene, which was cloned and sequenced, possesses a 4Fe-4S cluster, and is endowed with both *N*-glycosylase and AP lyase activities (*1*, *2*, *16*, *17*). The crystal structure of Nth-Eco was resolved (*18*), and its functional homologues were identified in eukaryotes (*19*−*22*). This enzyme possesses a broad substrate specificity for cytosine- and thymine-derived lesions in DNA (reviewed in refs *1* and *2*). These include uracil glycol (UraGly), 5-hydroxycytosine (5-OH-Cyt), 5-hydroxyuracil (5-OH-Ura), alloxan, 5-hydroxyhydantoin (5-OH-Hyd), urea, *trans*-1-carbamoyl-2-oxo-4,5-dihydroxyimidazolidine (*trans*-CODI), 5,6-dihydrouracil (5,6-diHUra), 5-hydroxy-6-hydrouracil (5-OH-6-HUra), 6-hydroxy-5-hydrouracil (6-OH-

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[1] Abbreviations: Nth-Eco, *E. coli* Nth protein (endonuclease III); GC/IDMS: gas chromatography/isotope-dilution mass spectrometry; asc, ascorbic acid; FapyAde, 4,6-diamino-5-formamidopyrimidine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 2-OH-Ade, 2-hydroxyadenine; 8-OH-Ade, 7,8-dihyro-8-oxoadenine, 8-hydroxyadenine; 8-OH-Gua, 7,8-dihyro-8-oxoguanine, 8-hydroxyguanine; hNTH1, human Nth protein; Nth-Spo, *Schizosaccharomyces pombe* Nth protein; 5,6-diOHCyt, 5,6-dihydroxycytosine; CytGly, cytosine glycol; UraGly, uracil glycol; 5-OH-Cyt, 5-hydroxycytosine; 5-OH-Ura, 5-hydroxyuracil; 5-OH-Hyd, 5-hydroxyhydantoin; 5,6-diHUra, 5,6-dihydrouracil; 5-OH-6-HUra, 5-hydroxy-6-hydrouracil, 6-OH-5-HUra, 6-hydroxy-5-hydrouracil; 6-OH-5-HCyt, 6-hydroxy-5-hydrocytosine; 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; 6-OH-5-HThy, 6-hydroxy-5-hydrothymine; *trans*-CODI, *trans*-1-carbamoyl-2-oxo-4,5-dihydroxyimidazolidine.

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5-HUra), 6-hydroxy-5-hydrocytosine (6-OH-5-HCyt), thymine glycol (ThyGly), 5,6-dihydrothymine (5,6-diHThy), 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd), 5-hydroxy-6-hydrothymine (5-OH-6-HThy), β-ureidoisobutyric acid, methyltartoronyl-*N*-urea and a ring fragmentation product of thymine C5-hydrate (5-OH-6-HThy). The uracil derivatives in DNA result from the deamination of the products of cytosine (*9*).

In most cases of substrate identification, the excision of only one or a small number of lesions was investigated at a time. This was due to the use of defined oligonucleotides containing a single lesion and/or to the limitation of the analytical techniques. In this work, we used gas chromatography/mass spectrometry (GC/MS), which permits the concurrent measurement of the products of all four DNA bases in a given DNA sample. For this reason, it enables the determination of substrate specificities of DNA repair enzymes, by precisely identifying which lesions are excised or not excised from damaged DNA containing a broad spectrum of lesions. This approach also facilitates the measurement of excision kinetics. The use of whole damaged DNA instead of defined oligonucleotides containing a single lesion provides a quantitative comparison of the substrate specificity of a DNA glycosylase for a multitude of modified bases under identical conditions.

The GC/MS technique was applied to the determination of substrate specificities and excision kinetics of various DNA glycosylases for modified bases in DNA damaged by free radicals (*8*, *9*, *23*−*29*). Recently, GC/MS was used to determine the excision of some cytosine- and thymine-derived lesions by *E. coli* Nth and Fpg proteins from γ-irradiated DNA and defined oligonucleotides (*11*, *30*, *31*). Thus far, however, the concurrent measurement of kinetics of excision of modified bases by the Nth-Eco from free radical-damaged DNA was not reported.

The objective of the present study was to explore whether, using several DNA substrates damaged by different free radical-generating systems, we may identify any lesions that had not been previously recognized as the substrates of Nth-Eco, when one type of damaged DNA substrate or defined oligonucleotides were used. Furthermore, we wished to investigate excision kinetics of Nth-Eco using free radical-damaged DNA, and thus to quantitatively compare its substrate specificity with those of its functional homologues from other organisms.

EXPERIMENTAL PROCEDURES

Materials.[2] Materials were obtained as described (*23*). The isolation and purification of the recombinant Nth-Eco were carried out as described (*9*). Two lots of Nth-Eco were prepared at different times. The enzyme was purified from an *E. coli fpg*$^-$ strain. During the purification procedure, Nth and Nei proteins were separated by several chromatographic steps. Other samples of Nth-Eco were obtained from Dr. B. Tudek (Polish Academy of Sciences, Warsaw, Poland), Dr. T. O'Connor (City of Hope Medical Center, Duarte, CA), and Drs. Y. W. Kow and P. W. Doetsch (Emory University, Atlanta, GA). All preparations of Nth-Eco were free of both *E. coli* Fpg and Nei proteins. *trans*-1-Carbamoyl-2-oxo-4,5-dihydroxyimidazolidine and its stable isotope-labeled analogue were gifts from Dr. J. R. Wagner (University of Sherbrooke, Québec, Canada).

Preparation of DNA Substrates. Calf thymus DNA (Sigma) was dissolved in 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 irradiated with γ-rays in a ^{60}Co γ-source at a dose of 80 Gy (dose rate 38.3 Gy/min). Another aliquot of the DNA solution was treated with H_2O_2 (final concentration 3 mM) in the presence of $FeCl_3$, EDTA, and ascorbic acid (final concentrations 20, 100, and 80 μM, respectively) for 1 h at 37 °C. $FeCl_3$, EDTA, and ascorbic acid were mixed before addition to the DNA solution. H_2O_2 was added last. Subsequently, untreated and treated DNA solutions were dialyzed against 10 mM phosphate buffer (pH 7.4) for 18 h. Phosphate buffer outside the dialysis tubes was changed three times during the course of dialysis.

Enzymatic Assays. Aliquots of DNA substrates (100 μg) were dried in a SpeedVac under vacuum and were then dissolved in 100 μL of the incubation buffer consisting of phosphate buffer (final concentration 50 mM, pH 7.4), 100 mM KCl, 1 mM EDTA, and 0.1 mM dithiothreitol. For the determination of the dependence of excision on the enzyme amount, 1, 2, 3, or 5 μg of Nth-Eco were added to the mixture and three replicates of each mixture were incubated at 37 °C for 30 min. Time dependence of excision was determined by incubation of the samples with 2 μg of Nth-Eco for 5, 10, 20, 30, 45, and 60 min. As controls, DNA samples were incubated with the deactivated enzyme or without the enzyme. Deactivation of the enzyme was achieved by heating at 140 °C for 15 min. After incubation, 260 μL of cold ethanol (−20 °C) were added to stop the reaction and precipitate DNA. The samples were kept at −20 °C for 2 h. Aliquots of stable isotope-labeled analogues of modified DNA bases and an additional 180 μL of cold ethanol (−20 °C) were added. The samples were centrifuged at 15000*g* for 30 min at 4 °C. DNA pellets and supernatant fractions were separated. Supernatant fractions were freed from ethanol in a SpeedVac under vacuum, frozen in liquid nitrogen and then lyophilized for 18 h.

For the measurement of excision kinetics, 10, 22, 35, 50, and 75 μg of damaged DNA were mixed with 90, 78, 65, 50, and 25 μg of undamaged DNA, respectively. Additional samples containing 100 μg of damaged or undamaged DNA were also used. Two sets of these samples with three replicates of each mixture were prepared. One set of samples was used to determine of the amounts of modified DNA bases in each sample. For this purpose, stable isotope-labeled analogues of modified bases as internal standards were added to the samples. Subsequently, they were dried in a SpeedVac under vacuum and hydrolyzed with 0.5 mL of 60% formic acid in evacuated and sealed tubes for 30 min at 140 °C. The hydrolysates were frozen in liquid nitrogen and lyophilized for 18 h.

The second set of samples was used for the measurement of the amounts of modified bases released by Nth-Eco. Three replicates of these samples were dried in a SpeedVac under

[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.

vacuum and then dissolved in 100 μL of the incubation buffer. The samples were incubated with or without 2 μg of Nth-Eco protein at 37 °C for 30 min. The amount of Nth-Eco corresponded to an enzyme concentration of 747 nM. After incubation, cold ethanol was added and then the samples were treated as described above for determination of enzyme amount and time dependence of excision.

Analysis by GC/IDMS. An aliquot (0.1 mL) of a mixture of nitrogen-bubbled bis(trimethylsilyl)trifluoroacetic acid [containing trimethylchlorosilane (1%; v/v)] and acetonitrile (4:1, v/v) was added to the lyophilized samples. The samples were vortexed and purged individually with ultra-high-purity nitrogen, tightly sealed under nitrogen with Teflon-coated septa, and then heated at 120 °C for 30 min. After cooling, the samples, which had been treated with the enzyme, were centrifuged at 5000*g* for 30 min to precipitate the salt. The clear supernatant fractions were removed and placed in vials used for injection of samples onto the GC-column. Vials were purged with nitrogen and tightly sealed with septa. Aliquots (4 μL) of derivatized samples were analyzed by GC/IDMS with selected-ion monitoring under the experimental conditions described previously (*23*). The oven temperature of the gas chromatograph was programmed from 130 to 280 °C at a rate of 8 °C/min after 2 min at 130 °C.

RESULTS

Modified Bases in DNA Substrates. We used three different types of damaged DNA samples to look for substrates of Nth-Eco that had not been recognized previously and to investigate the excision kinetics of this enzyme. DNA samples were prepared by γ-irradiation in air- or N_2O-saturated aqueous solution or by treatment with H_2O_2/Fe(III)-EDTA/asc. Using GC/IDMS, the following modified bases were identified and quantified in these samples: 5-OH-Cyt, 5-OH-Ura, 5-OH-Hyd, isodialuric acid [identified as its enol form 5,6-dihydroxyuracil (5,6-diOH-Ura)], 5,6-diHUra, 5-OH-6-HUra, t*rans*-CODI, 5,6-diHThy, 5-OH-5-MeHyd, 5-OH-6-HThy, 5-(hydroxymethyl)uracil, ThyGly, 4,6-diamino-5-formamidopyrimidine (FapyAde), 8-hydroxyadenine (8-OH-Ade), 2-hydroxyadenine (2-OH-Ade), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), and 8-hydroxyguanine (8-OH-Gua). These compounds are produced in DNA by reactions of hydroxyl radicals, except for 5,6-diHThy and 5,6-diHUra (or 5,6-diHCyt), which result from reactions of radiation-generated H atoms (reviewed in refs *32*−*34*). It is known that 5-OH-Cyt and 5-OH-Ura are formed by acid-induced modification of cytosine glycol (CytGly), the former by dehydration and the latter by deamination and dehydration (*9*, *32*, *33*). CytGly was detected by GC/MS as a hydroxyl radical-induced product of cytosine in aqueous solution (*9*). Similarly, the other uracil derivatives listed are known to be products of cytosine and result from spontaneous and/or acid-induced deamination of their corresponding cytosine derivatives (*9*, *32*−*34*). 5,6-diHUra, 5-OH-6-HUra, 5,6-diHThy, and 5-OH-6-HThy were not formed in DNA irradiated under air or treated with H_2O_2/Fe(III)-EDTA/asc. Oxygen inhibits the formation of these compounds (reviewed in ref *33*). 5-OH-Hyd is known to be produced by acid-induced decarboxylation of alloxan [(2,4,5,6-(1H,3H)-pyrimidinetetrone], which in turn is formed by spontaneous oxidation of dialuric acid in aerated aqueous solution (*9*, *35*, *36*). It is also possible that 5-OH-Hyd is formed in DNA by a mechanism, which does not involve alloxan (*33*). Formation of a small amount of *trans*-CODI was observed in agreement with previous studies (*11*, *37*). This compound is a major product of cytosine irradiated in aqueous solution (*32*, *37*); however, it is formed in DNA only with a relatively low yield compared to other products (*11*, *37*).

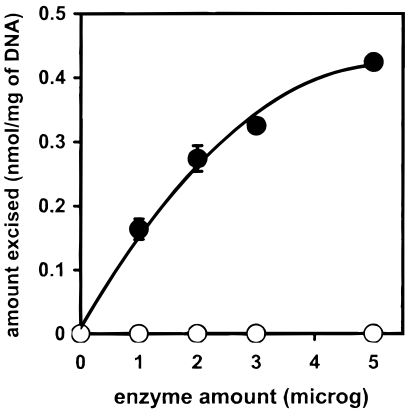

FIGURE 1: Excision of FapyAde by Nth-Eco as a function of the enzyme amount. DNA γ-irradiated under N_2O (100 μg) was used as a substrate. The incubation time was 30 min at 37 °C. The amounts given on the *y*-axis represent those found in the supernatant fractions. One nanomole of a lesion/mg of DNA corresponds to ~32 lesions/10^5 DNA bases. Closed circles, incubation with active enzyme; open circles, incubation with deactivated enzyme.

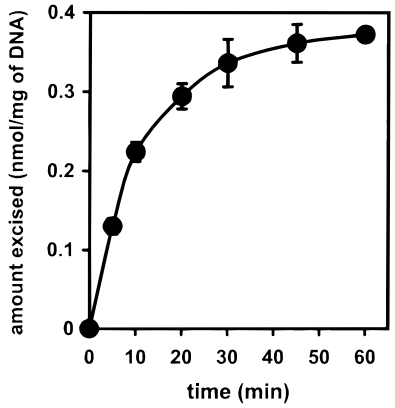

FIGURE 2: Excision of FapyAde by Nth-Eco as a function of incubation time. DNA γ-irradiated under N_2O (100 μg) was used as a substrate. The incubation was at 37 °C. The enzyme amount was 2 μg/100 μg of DNA. The amounts given on the *y*-axis represent those found in the supernatant fractions. One nmol of a lesion/mg of DNA corresponds to ~32 lesions/10^5 DNA bases.

Novel Substrates of E. coli Nth Protein. We observed the excision of FapyAde, 5,6-diOH-Ura, and 5,6-dihydroxycytosine (5,6-diOH-Cyt) from damaged DNA substrates in addition to known substrates of Nth-Eco such as 5-OH-Cyt, 5-OH-Ura, *cis*- and *trans*-uracil glycols, alloxan, 5,6-diHUra, 5-OH-6-HUra, t*rans*-CODI, 5,6-diHThy, 5-OH-5-MeHyd, 5-OH-6-HThy, and *cis*- and *trans*-thymine glycols. As examples, Figures 1 and 2 illustrate the excision of FapyAde as a function of enzyme amount and incubation time, respectively, from DNA irradiated under N_2O. No excision was observed when deactivated enzyme was used (Figure 1). We also conducted experiments using four separately prepared samples of Nth-Eco from four different laboratories to ascertain that the excision of FapyAde, 5,6-diOH-Ura, and 5,6-diOH-Cyt was not an artifact of a specific enzyme

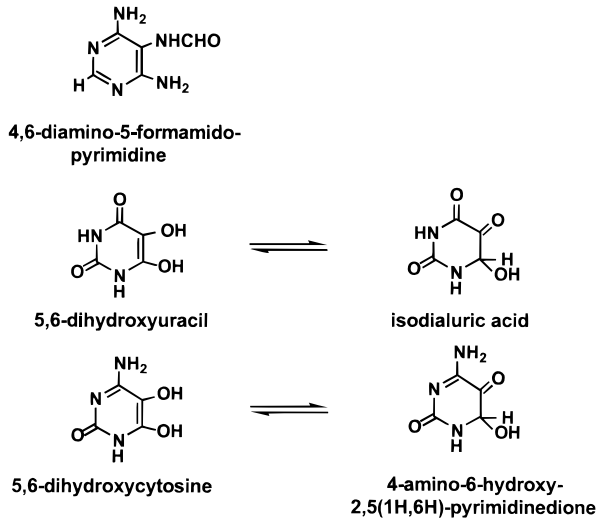

FIGURE 3: Structures of the novel substrates of Nth-Eco.

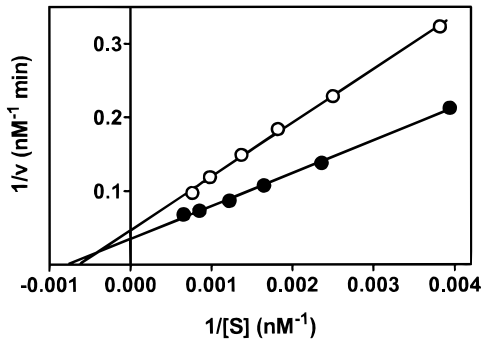

FIGURE 4: Lineweaver-Burk plots of excision of FapyAde (closed circles) from DNA irradiated under N_2O and of excision of 5,6-diOH-Cyt (open circles) from DNA irradiated under air. The incubation time was 30 min at 37 °C. The enzyme amount was 2 μg/100 μg of DNA. S, concentration of FapyAde or 5,6-diOH-Cyt; v, initial velocity. The amounts of products found in supernatant fractions were used for initial velocity. The amounts of 5,6-diOH-Cyt represent the sum of 5,6-diOH-Cyt, and 5,6-diOH-Ura.

preparation. These four Nth-Eco samples also excised FapyAde from all three DNA substrates, and 5,6-diOH-Ura and 5,6-diOH-Cyt from DNA irradiated under air and DNA treated with H_2O_2/Fe(III)-EDTA/asc. The excision of 5,6-diOH-Ura and 5,6-diOH-Cyt from DNA irradiated under N_2O was not significant in agreement with a previous study (*9*). Figure 3 illustrates the structures of FapyAde, 5,6-diOH-Ura, and 5,6-diOH-Cyt. It should be pointed out that 5,6-diOH-Ura exists in its keto form isodialuric acid (*35*) (Figure 3), which enolyzes upon derivatization for GC/MS analysis (*9*). Isodialuric acid was identified as a hydroxyl radical-induced product of cytosine moiety in DNA and related cytosine compounds (*9*, *34*, *38*). It is not known whether isodialuric acid actually existed in DNA prior to enzymatic treatment and was excised by the enzyme or arose during workup from partial deamination of 5,6-diOH-Cyt, which had previously been identified as a hydroxyl radical-induced product of cytosine and cytosine moiety in DNA (*9*, *32*, *39*, *40*). Similar to isodialuric acid, 5,6-diOH-Cyt may exist in its keto form 4-amino-6-hydroxy-2,5(1H,6H)-pyrimidinedione (Figure 3). Recently, 5,6-diOH-Cyt was shown to be a substrate of *Schizosaccharomyces pombe* Nth protein (Nth-Spo) and human Nth protein (hNTH1) (*26*, *28*). We observed excision of alloxan in agreement with a previous study (*9*). However, no excision of 5-OH-Hyd was observed. This compound that was found in acid-hydrolysates of DNA samples arises from acid-induced decarboxylation of alloxan (*9*, *35*, *36*). This suggests that alloxan did not decarboxylate during enzymatic digestion or thereafter during workup under our experimental conditions. In another study, 5-OH-Hyd, not alloxan, was excised by Nth-Eco from irradiated DNA (*11*). Alloxan may have been converted to 5-OH-Hyd under those conditions even in neutral solution. This assumption is supported by the fact that both alloxan and 5-OH-Hyd were recently identified as products of 2′-deoxycytidine damaged by the Fenton reaction (*38*).

Cis- and *trans*-uracil glycols, 5-OH-Ura and 5-OH-Cyt were excised from all three DNA substrates. It is not known, however, whether the enzyme actually excised these compounds or they were formed by modification of excised *cis*- and *trans*-cytosine glycols during workup. Cytosine glycols were not detected in supernatant fractions in this work. Excision of uracil glycol (possibly *cis*-isomer), 5-OH-Ura, and 5-OH-Cyt was reported from oligonucleotides with a single lesion embedded in a defined position (*10*, *14*, *31*, *41*). This suggests that these compounds are substrates of Nth-Eco and are likely to be excised from DNA as well. 5-OHMeUra, 8-OH-Ade, 2-OH-Ade, FapyGua, and 8-OH-Gua were not excised significantly from any of the DNA substrates.

Excision Kinetics. Excision kinetics was determined by varying the concentration of the base products. Concentration ranges were similar to those previously used for determination of excision kinetics of other DNA glycosylases (*23*, *26*, *27*). The measured amounts of excised products in supernatant fractions were used for this purpose. Excision followed Michaelis−Menten kinetics (*42*). Kinetic constants and standard deviations ($n = 6$) were calculated using Lineweaver−Burk plots (*42*) and a linear least-squares analysis of the data. Initial velocities were estimated by using the plots of excision as a function of incubation time. As examples, Figure 4 illustrates Lineweaver−Burk plots of excision of FapyAde and 5,6-diOH-Cyt. Kinetic parameters are given in Tables 1−3. Tables 4 and 5 also show a comparison of the specificity constants (k_{cat}/K_M) of Nth-Eco with those of hNTH1, Nth-Spo and *S. cerevisiae* Ntg1 and Ntg2 proteins for the same modified DNA bases. Kinetic parameters of excision of 5,6-diOH-Cyt and 5,6-diOH-Ura are given in the tables as a sum, because 5,6-diOH-Ura and 5-OH-Ura only were detected in acid hydrolysates of DNA samples, due to complete acid-induced deamination of 5,6-diOH-Cyt. This is also true for the kinetic parameters of UraGly and 5-OH-Ura because of the complete dehydration of UraGly. The ratio of the amount of 5,6-diOH-Ura to that of 5,6-diOH-Cyt in supernatant fractions of two DNA substrates was ~3.4. The ratio of the amount of uracil glycol to that of 5-OH-Ura was ~2−4, depending on the DNA substrate. Kinetic parameters of excision of 5,6-diHThy, 5,6-diHUra, and *trans*-CODI could not be determined because of the low extent of excision from all three DNA substrates.

DISCUSSION

We discovered three new substrates of Nth-Eco and studied their kinetics of excision along with those of a

Table 1: Maximum Velocities [V_{max} (nM × min^{-1})[a]] for Excision of Modified Bases by Eco-Nth from DNA Treated with Various Free Radical-Generating Systems

substrate	irradiation (N_2O)	irradiation (air)	H_2O_2/Fe(III)-EDTA/asc
FapyAde	28.3 ± 1.8[b,f−h,k]	8.8 ± 0.3[c,d−g,i]	21.6 ± 1.8[d−i]
ThyGly	27.6 ± 1.7[b,c,e−h,k]	41.1 ± 7.3[c,e,f,h,i]	73.2 ± 8.0[f,h]
5-OH-Cyt	16.0 ± 1.6[c,g,h]	23.3 ± 2.4[c,g,h]	64.7 ± 4.3
UraGly	14.2 ± 1.2[b,c]	25.0 ± 2.2[g,h]	38.9 ± 3.6[i]
5-OH-5-MeHyd	9.2 ± 1.5[b,c,j]	41.2 ± 1.9[h,i]	51.7 ± 7.2
alloxan	8.9 ± 2.7[c,j]	7.6 ± 0.8[i]	35.7 ± 5.1[i]
5,6-diOH-Cyt[l]		21.3 ± 1.4	70.1 ± 4.5
5-OH-6-HThy	17.4 ± 0.4		
5-OH-6-HUra	10.6 ± 1.1		

[a] Values represent the mean ± standard deviation ($n = 6$). [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 line 2 ($P < 0.05$). [e] Statistically different from the value in line 3 ($P < 0.05$). [f] Statistically different from the value in line 4 ($P < 0.05$). [g] Statistically different from the value in line 5 ($P < 0.05$). [h] Statistically different from the value in line 6 ($P < 0.05$). [i] Statistically different from the value in line 7 ($P < 0.05$). [j] Statistically different from the value in line 8 ($P < 0.05$). [k] Statistically different from the value in line 9 ($P < 0.05$). The values of UraGly and 5,6-diOH-Cyt represent the sum of UraGly and 5-OH-Ura, and the sum of 5,6-diOH-Cyt and 5,6-diOH-Ura, respectively. [l] No significant excision of 5,6-diOH-Cyt (or 5,6-diOH-Ura) was observed from DNA irradiated under N_2O in agreement with a previous study (*9*).

Table 2: Michaelis Contants [K_M (nM)[a]] for Excision of Modified Bases by Nth-Eco from DNA Treated with Various Free Radical-Generating Systems

substrate	irradiation (N_2O)	irradiation (air)	H_2O_2/Fe(III)-EDTA/asc
FapyAde	1264 ± 86[b,c,f−h]	343 ± 18[c,d−i]	2149 ± 193[d−f,h,i]
ThyGly	1654 ± 104[c,e−k]	2832 ± 577[c,e,g−i]	5733 ± 711[e]
5-OH-Cyt	863 ± 95[c,g]	610 ± 102[c,f−i]	6804 ± 459[g]
UraGly	513 ± 51[b,c,j,k]	2028 ± 202[c,g]	5240 ± 511
5-OH-5-MeHyd	444 ± 78[b,c,j,k]	1144 ± 57[c]	3428 ± 513
alloxan	624 ± 204[b,c]	1375 ± 167[c]	5800 ± 844
5,6-diOH-Cyt		1554 ± 104[c]	5617 ± 380
5-OH-6-HThy	930 ± 30		
5-OH-6-HUra	978 ± 113		

[a] Values represent the mean ± standard deviation ($n = 6$). [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 line 2 ($P < 0.05$). [e] Statistically different from the value in line 3 ($P < 0.05$). [f] Statistically different from the value in line 4 ($P < 0.05$). [g] Statistically different from the value in line 5 ($P < 0.05$). [h] Statistically different from the value in line 6 ($P < 0.05$). [i] Statistically different from the value in line 7 ($P < 0.05$). [j] Statistically different from the value in line 8 ($P < 0.05$). [k] Statistically different from the value in line 9 ($P < 0.05$). The values of UraGly and 5,6-diOH-Cyt represent the sum of UraGly and 5-OH-Ura, and the sum of 5,6-diOH-Cyt and 5,6-diOH-Ura, respectively.

multitude of other modified bases from free radical-damaged DNA. These are FapyAde, 5,6-diOH-Ura, and 5,6-diOH-Cyt. This is the first time that a purine-derived lesion, FapyAde, was shown to be a substrate of Nth-Eco. Other purine derivatives, FapyGua, 8-OH-Gua, 8-OH-Ade, and 2-OH-Ade were not excised. FapyAde is also a substrate of *E. coli* Fpg protein (reviewed in refs *1* and *2*). This enzyme, however, excises FapyAde, FapyGua, and 8-OH-Gua from DNA with similar specificities (*23*). Thus, the contamination of Nth-Eco samples used in this work with Fpg protein is ruled out as expected, since Nth-Eco was purified from an *E. coli fpg*$^-$ strain.

Table 3: Specificity Contants [$k_{cat}/K_M \times 10^5$ (min^{-1} × nM^{-1})[a]] for Excision of Modified Bases by Nth-Eco from DNA Treated with Various Free Radical-Generating Systems

substrate	irradiation (N_2O)	irradiation (air)	H_2O_2/Fe(III)-EDTA/asc
FapyAde	3.1 ± 0.3[c,k]	3.5 ± 0.2[c,f,h]	1.4 ± 0.2[h]
ThyGly	2.3 ± 0.2	2.0 ± 0.5[e,g,h]	1.8 ± 0.3[h]
5-OH-Cyt	2.5 ± 0.4[b,c]	5.2 ± 0.9[c,f,h,i]	1.3 ± 0.1
UraGly	3.8 ± 0.5[b,c]	1.7 ± 0.2[g,h]	1.0 ± 0.15[g]
5-OH-5-MeHyd	2.8 ± 0.7	4.9 ± 0.3[c,h,i]	2.1 ± 0.4[h]
alloxan	1.9 ± 0.9[b,c]	0.8 ± 0.1[i]	0.8 ± 0.2[i]
5,6-diOH-Cyt		1.9 ± 0.2	1.7 ± 0.2
5-OH-6-HThy	2.6 ± 0.1		
5-OH-6-HUra	1.5 ± 0.2		

[a] Values represent the mean ± standard deviation ($n = 6$). ($k_{cat} = V_{max}$/[enzyme]) ([Nth-Eco] = 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 line 2 ($P < 0.05$). [e] Statistically different from the value in line 3 ($P < 0.05$). [f] Statistically different from the value in line 4 ($P < 0.05$). [g] Statistically different from the value in line 5 ($P < 0.05$). [h] Statistically different from the value in line 6 ($P < 0.05$). [i] Statistically different from the value in line 7 ($P < 0.05$). [k] Statistically different from the value in line 9 ($P < 0.05$). The values of UraGly and 5,6-diOH-Cyt represent the sum of UraGly and 5-OH-Ura, and the sum of 5,6-diOH-Cyt and 5,6-diOH-Ura, respectively.

We established kinetics of excision of a multitude of modified bases by Nth-Eco. This study differs from previous studies on excision kinetics of Nth-Eco in that they mostly used oligonucleotides with a single modified base embedded in a defined position. The use of DNA substrates enabled the simultaneous measurement of excision kinetics of Nth-Eco for a large number of modified bases under identical conditions. These measurements also facilitated a comparison of excision kinetics of Nth-Eco with those of its functional homologues (such as Nth-Spo, hNTH1, and *S. cerevisiae* Ntg1 and Ntg2 proteins) that had been determined under similar experimental conditions (*26*−*28*). This comparison clearly shows that there exist significant differences with respect to substrate specificity and kinetic parameters despite extensive structural conservation among the Nth homologues.

The results show that the preference of Nth-Eco for FapyAde is similar to those of other well-known substrates such as ThyGly and 5-OH-Cyt. FapyAde is also a substrate of *S. cerevisiae* Ntg1 and Ntg2 proteins (*27*). The k_{cat}/K_M values of FapyAde excision by these three enzymes from one type of DNA substrate were similar to one another, indicating a similar preference of these enzymes for FapyAde. The specificity constant of excision of the sum of 5,6-diOH-Ura and 5,6-diOH-Cyt was similar to those of ThyGly and UraGly and approximately 2-fold smaller than those of FapyAde, 5-OH-Cyt, and 5-OH-5-MeHyd in the case of DNA irradiated under air. The k_{cat}/K_M value for excision of 5,6-diOH-Ura and 5,6-diOH-Cyt from H_2O_2/Fe-EDTA/asc-treated DNA was similar to those of other lesions. These results indicate that 5,6-diOH-Ura and 5,6-diOH-Cyt are among the major substrates of Nth-Eco. A comparison with excision kinetics of Nth-Spo shows that both Nth-Eco and Nth-Spo excised 5,6-diOH-Ura and 5,6-diOH-Cyt with a similar preference from both DNA substrates. No significant excision of these compounds from DNA irradiated under N_2O was observed. This may be due to their low level in this DNA substrate in comparison with other substrates competing for excision by Nth-Eco or to unknown factors.

Table 4: Comparison of the Specificity Contants [$k_{cat}/K_M \times 10^5$ ($min^{-1} \times nM^{-1}$)[a]] for Excision of Modified Bases by Nth-Eco, hNTH1 (*28*), and Nth-Spo (*26*) from DNA Treated with Various Free Radical-Generating Systems

	irradiation (N_2O)			irradiation (air)			H_2O_2/Fe(III)-EDTA/asc		
substrate	Nth-Eco	hNTH1	Nth-Spo	Nth-Eco	hNTH1	Nth-Spo	Nth-Eco	hNTH1	Nth-Spo
FapyAde	3.1 ± 0.3			3.5 ± 0.2			1.4 ± 0.2		
ThyGly	2.3 ± 0.2	1.6 ± 0.3	2.2 ± 0.4	2.0 ± 0.5	1.5 ± 0.3	1.5 ± 0.1	1.8 ± 0.3	1.3 ± 0.1	1.1 ± 0.1
5-OH-Cyt	2.5 ± 0.4	3.5 ± 0.6	4.4 ± 0.1	5.2 ± 0.9[c]	3.7 ± 0.4	2.5 ± 0.2	1.3 ± 0.1[b]	2.4 ± 0.2[c]	1.1 ± 0.1
5-OH-Ura	3.8 ± 0.5		3.4 ± 0.3	1.7 ± 0.2	1.6 ± 0.4	1.4 ± 0.06	1.0 ± 0.15	1.0 ± 0.2	1.5 ± 0.2
5-OH-5-MeHyd	2.8 ± 0.7			4.9 ± 0.3			2.1 ± 0.4		
alloxan	1.9 ± 0.9			0.8 ± 0.1			0.8 ± 0.2		
5,6-diOH-Cyt			3.3 ± 0.5	1.9 ± 0.2		1.9 ± 0.3	1.7 ± 0.2		1.6 ± 0.15
5-OH-6-HThy	2.6 ± 0.1	2.7 ± 0.2	2.0 ± 0.2						
5-OH-6-HUra	1.5 ± 0.2								

[a] Values represent the mean ± standard deviation ($n = 6$). ($k_{cat} = V_{max}$/[enzyme]). ([Nth-Eco] = 757 nM, [hNTH1] = 530 nM, [Nth-Spo] = 452 nM. [b] Statistically different from the value in column 2 ($P < 0.05$). [c] Statistically different from the value in column 3 ($P < 0.05$). In the case of Nth-Eco, the values of 5-OH-Ura and 5,6-diOH-Cyt represent the sum of UraGly and 5-OH-Ura, and the sum of 5,6-diOH-Cyt and 5,6-diOH-Ura, respectively.

Table 5: Comparison of the Specificity Contants [$k_{cat}/K_M \times 10^5$ ($min^{-1} \times nM^{-1}$)[a]] for Excision of Modified Bases by Nth-Eco and *S. cerevisiae* Ntg1 and Ntg2 Proteins (*27*) from DNA γ-Irradiated under N_2O

substrate	Nth-Eco	Ntg1	Ntg2
FapyAde	3.1 ± 0.3	4.9 ± 0.7	4.7 ± 0.5
ThyGly	2.3 ± 0.2	2.2 ± 0.3	1.6 ± 0.1
5-OH-Cyt	2.5 ± 0.4	4.1 ± 0.9	4.2 ± 0.2
5-OH-Ura	3.8 ± 0.5	5.1 ± 0.8	5.5 ± 0.5
5-OH-5-MeHyd	2.8 ± 0.7	3.3 ± 0.5	
alloxan	1.9 ± 0.9		
5,6-diOH-Cyt			
5-OH-6-HThy	2.6 ± 0.1[b,c]	15.0 ±0.6	22.7 ± 1.0
5-OH-6-HUra	1.5 ± 0.2[b,c]	8.8 ± 2.7	7.8 ± 0.8

[a] Values represent the mean ± standard deviation ($n = 6$). ([Nth-Eco] = 757 nM; [Ntg1] = 395 nM; [Ntg2] = 413 nM). [b] Statistically different from the value in column 2 ($P < 0.05$). [c] Statistically different from the value in column 3 ($P < 0.05$). Other statistical differences are given in Table 4 and elsewhere (*27*). In the case of Nth-Eco, the value of 5-OH-Ura represents the sum of UraGly and 5-OH-Ura.

Their levels in the other two DNA substrates were 6- to 12-fold greater.

The k_{cat}/K_M values for the excision of lesions from DNA irradiated under N_2O or from H_2O_2/Fe(III)-EDTA/asc-treated DNA were similar, pointing to a similar preference of Nth-Eco for all substrates within a DNA substrate, except for alloxan with the lowest specificity constant in the latter case. In the case of DNA irradiated under air, there were significant differences between the specificity constants. Significant differences were also noted between DNA substrates in terms of the preference for the same lesion. These results clearly indicate a dependence of excision on the nature of the damaged DNA substrate. This phenomenon has also been observed with other DNA glycosylases (*23*, *25*, *26*, *28*, *29*). In the same context, it is interesting to note that the K_M values of excision of 5-OH-Cyt, ThyGly, and UraGly from irradiated DNA in this work were somewhat similar to those of excision of these compounds from defined oligonucleotides (*31*, *41*). In one case, the K_M value of 5-OH-Cyt excision from an oligo-33-mer (*31*) was significantly greater than that from an oligo-40-mer (*41*) and those from irradiated DNA substrates, but similar to that from H_2O_2/Fe(III)-EDTA/asc-treated DNA in this work. On the other hand, another paper reported much smaller K_M values for excision of ThyGly and UraGly from an oligo-18-mer (*14*). It appears that there exists a discrepancy between the results obtained with different types of oligomers. On the other hand, this may indicate that the nature of an oligomer may play a role in excision kinetics similar to that seen with DNA substrates.

5-OH-6-HThy and 5-OH-6-HUra were excised by Nth-Eco with a similar preference to other substrates, indicating that they are among the major substrates of Nth-Eco. The efficient excision of 5-OH-6HThy from DNA irradiated under N_2O is in contrast to its rather low excision from DNA irradiated under nitrogen and in the presence of cysteine (*30*). 5-OH-6-HThy was excised by Nth-Eco, Nth-Spo, and hNTH1 with a similar preference. Kinetic parameters of 5-OH-6-HUra for excision by the latter two enzymes were not reported (*26*, *28*). On the other hand, 5-OH-6-HThy and 5-OH-6-HUra were excised with approximately 5−8-fold greater preference by *S. cerevisiae* Ntg1 and Ntg2 proteins than by Nth-Eco. 5,6-diHThy and 5,6-diHUra that are also specific to DNA irradiated in the absence of oxygen were excised to such a small extent that no excision rates could be given. The low excision of 5,6-diHThy from DNA substrates is in agreement with two recent reports (*30*, *31*).

5-OH-Cyt was excised efficiently from all three DNA substrates. This is in agreement with a previous study that showed significant excision of this compound from DNA irradiated under air, albeit reporting no Michaelis−Menten kinetics (*11*). Its excision kinetics was similar to those reported for the functional homologues of Nth-Eco from other organisms (*26*−*28*). 5-OH-Cyt may have existed in DNA prior to enzymatic digestion and excised by Nth-Eco. On the other hand, 5-OH-Cyt along with 5-OH-Ura and UraGly may have been formed from CytGly (*9*, *33*) following enzymatic digestion. The detection of UraGly in supernatant fractions clearly shows the presence of CytGly in damaged DNA at some point prior to enzymatic digestion. 5-OH-Cyt, 5-OH-Ura, and UraGly are excised by Nth-Eco from defined oligonucleotides, indicating that they are among the major substrates of this enzyme (*10*, *14*, *31*, *41*). A recent study reported a low detection level of 5-OH-Cyt in supernatant fractions of defined oligonucleotides when GC/MS was used, and this was thought to result from a chemical change of 5-OH-Cyt (*31*). This is in contrast to the facile detection of 5-OH-Cyt in supernatant fractions in the present study and elsewhere (*11*) and as a major substrate of the functional homologues of Nth-Eco (*26*−*28*). 5-OH-Cyt is a stable compound, and thus it is not expected to undergo any change during mild enzymatic hydrolysis. The V_{max} values

of the excision of 5-OH-Cyt from all three DNA substrates, which were similar to those of other major substrates of Nth-Eco, attest to this fact. The prepurification procedure used in the previous study (*31*) to purify supernatant fractions may have caused the loss of 5-OH-Cyt. We reported (*23*−*29*) and confirmed in this work that a prepurification of enzymatic hydrolysates of DNA prior to GC/IDMS analysis (*30*, *31*, *43*) is unnecessary for precise measurement of modified bases excised by a given DNA glycosylase. We found that modified bases and their stable isotope-labeled analogues used as internal standards are completely recovered by our procedure, rendering prepurification unnecessary.

In conclusion, we discovered three additional substrates of Nth-Eco, one of which is a purine-derived compound. We also established excision kinetics of Nth-Eco for a multitude of modified DNA bases. The new substrates were excised with a similar preference to well-known major substrates of Nth-Eco. The results facilitated a comparison of the substrate specificity and kinetic parameters of Nth-Eco with those of its functional homologues from other organisms, pointing to major differences despite extensive structural conservation among Nth homologues. This study extends the substrate specificity of Nth-Eco and reports simultaneously measured kinetics of excision of a number of modified bases from DNA for the first time.

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