Stimulation of DNA Glycosylase Activity of OGG1 by NEIL1: Functional Collaboration between Two Human DNA Glycosylases†

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ABSTRACT: The eukaryotic 8-oxoguanine−DNA glycosylase 1 (OGG1) provides the major activity for repairing mutagenic 7,8-dihydro-8-oxoguanine (8-oxoG) induced in the genome due to oxidative stress. Earlier in vitro studies showed that, after excising the base lesion, the human OGG1 remains bound to the resulting abasic (AP) site in DNA and does not turn over efficiently. The human AP-endonuclease (APE1), which cleaves the phosphodiester bond 5′ to the AP site, in the next step of repair, displaces the bound OGG1 and thus increases its turnover. Here we show that NEIL1, a DNA glycosylase/AP lyase specific for many oxidized bases but with weak 8-oxoG excision activity, stimulates turnover of OGG1 in a fashion similar to that of APE1 and carries out $\beta\delta$-elimination at the AP site. This novel collaboration of two DNA glycosylases, which do not stably interact with each other, in stimulating 8-oxoguanine repair is possible because of higher AP site affinity and stronger AP lyase activity of NEIL1 relative to OGG1. Comparable levels of NEIL1 and OGG1 in some human cells raise the possibility that NEIL1 serves as a backup enzyme to APE1 in stimulating 8-oxoG repair in vivo.

Cellular DNA is being damaged continuously by reactive oxygen species (ROS) that are generated as byproducts of respiration and are due to other extrinsic factors. 7,8-Dihydro-8-oxoguanine (8-oxoG),[1] one of the most abundant DNA lesions generated by reaction of guanine with ROS (*1*), is mutagenic because of its propensity to mispair with A during DNA synthesis (*2*, *3*). This and most other oxidized base lesions in DNA are repaired predominantly via the base excision repair (BER) pathway in which repair is initiated with excision of the damaged base by a DNA *N*-glycosylase. The phosphodiester bond 5′ to the AP site generated after removal of the base lesion in DNA is cleaved generally by an AP-endonuclease (APE) in the next step of repair. In mammalian cells, 8-oxoguanine−DNA glycosylase (OGG1) and an endonuclease III homologue (NTH1) were characterized to be the major oxidatively damaged base-specific DNA glycosylases which, like their counterparts in bacteria and yeast, possess intrinsic AP lyase activity. As a result, these enzymes, after excising the damaged base, carry out a β-elimination reaction at the resulting AP site and cleave the phosphodiester backbone to generate 3′ α,β-unsaturated phosphodeoxyribose and 5′-phosphate termini (*4*−*6*).

We and others have shown earlier that the human OGG1 possesses weak AP lyase activity and inefficiently carries out β-elimination at the AP site. It also turns over poorly because of its strong affinity for the AP site generated after excision of 8-oxoG from DNA (*7*, *8*). APE1, the primary mammalian APE, strongly enhances OGG1 activity by competing for the AP site and displacing OGG1 (*7*). These results are consistent with the "hand-off model" proposed for enhanced efficiency in BER in which the product of the one enzyme is sequestered by remaining bound to the enzyme until the next enzyme in the pathway acts on this substrate (*9*, *10*). The binding of OGG1 to its own reaction product may be physiologically significant for preventing the side reaction of the unstable AP site including premature cleavage. Similar product inhibition has also been observed for other mono- and bifunctional DNA glycosylases (*11*−*15*). Although OGG1 does not stably interact with APE1 (*7*), other DNA glycosylases, e.g., G·T thymine−DNA glycosylase (TDG), were found to form a stable complex with APE1 (*14*).

We and others have recently characterized two new oxidized base-specific DNA glycosylases, which belong to the FPG/Nei family, and named them NEIL1 and NEIL2 (*16*−*21*). These enzymes possess intrinsic AP lyase activity but, unlike OGG1 and NTH1, carry out $\beta\delta$-elimination at an AP site in DNA and generate 3′-phosphate and 5′-phosphate termini with removal of the deoxyribose residue. NEIL1 has weak 8-oxoG excision activity relative to its other substrates such as 5-hydroxyuracil (5-OHU) or thymine glycol (*16*, *19*). Here we report that OGG1 is stimulated by NEIL1 in a similar manner as by APE1. To our knowledge, this is the first documentation of functional interaction between two human DNA glycosylases which has significant implication about the novel role of NEIL1 in BER.

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[1] Abbreviations: AP, abasic; APE, AP-endonuclease; BER, base excision repair; 8-oxoG, 7,8-dihydro-8-oxoguanine; EMSA, electrophoretic mobility shift assay; Fpg, FapyG−DNA glycosylase; 5-OHU, 5-hydroxyuracil; NEIL, Nei-like; OGG1, 8-oxoguanine−DNA glycosylase; ROS, reactive oxygen species; TBE, Tris/borate/EDTA; TDG, T·G-specific thymine−DNA glycosylase; TG, thymine glycol; THF, tetrahydrofuran; Udg, uracil−DNA glycosylase; WT, wild type.

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EXPERIMENTAL PROCEDURES

Proteins. Expression and purification of the recombinant human wild-type (WT) and mutant OGG1 (K249Q) and WT NEIL1 were described earlier (*7*, *16*). Previous studies from our laboratory have shown that mutation of the conserved Lys-53 residue in NEIL1 (K53L) abolishes both glycosylase (*22*) and AP lyase activity (T. K. Hazra, unpublished results), unlike in the case of Fpg/Nei (*22*). The K53L mutant of NEIL1 was expressed as a C-terminal His-tag fusion polypeptide from a recombinant pET22b vector constructed by using a site-directed mutagenesis kit (Stratagene). The NEIL1 mutant protein was purified by affinity chromatography on a Ni^{2+} column followed by chromatography in a HiTrap-SP column (*22*). *Escherichia coli* FapyG−DNA glycosylase (Fpg) and uracil−DNA glycosylase (Udg) were purchased from New England Biolabs.

Preparation of Oligonucleotide Substrates. The 31-mer oligonucleotides with the sequence 5′-GAA GAG AGA AAG AGA XAA GGA AAG AGA GAA G-3′, in which 8-oxoG or 5-OHU or tetrahydrofuran (THF) (indicated by X) was incorporated at position 16 and used in earlier studies (*7*), were purchased from Midland Co. The oligo was labeled at the 5′ end using [γ-^{32}P]ATP (Amersham Biosciences) and T4 polynucleotide kinase (New England Biolabs) and then annealed to an oligo with the complementary sequence at a 1:1 molar ratio. To obtain a duplex DNA containing an AP site and 3′-phosphate product, a duplex oligo with a U·C pair in an oligo of the same sequence as the lesion-containing oligos but with U at position 16 was treated with 1 unit of Udg or Udg together with 5 units of Fpg, respectively, followed by phenol/chloroform extraction and ethanol precipitation.

Enzyme Assays. Enzyme activities were measured in a buffer containing 25 mM HEPES, pH 7.9, 1 mM DTT, 5% glycerol, 50 mM KCl, 0.1 μg/μL bovine serum albumin, and 1 mM EDTA at 37 °C. The reaction mixture (300 μL) for single-turnover assays contained 2.0 nM 5′-^{32}P-labeled 31-mer duplex 8-oxoG·C containing oligo. To measure AP lyase activity, 10 μL aliquots removed at various times were immediately mixed with an equal volume of loading dye containing SDS and glycerol and electrophoresed in denaturing polyacrylamide gel. Under these conditions, the AP sites are not spontaneously cleaved. To measure base excision activity, 10 μL aliquots were removed and treated with an equal volume of a mixture containing 95% formamide, 10 mM EDTA, 0.05% xylene cyanol, and 200 mM piperidine and then heated at 95 °C for 5 min to ensure complete strand cleavage at the AP site (*7*). Analysis of OGG1 activity under multiple-turnover condition was performed in the same way except that the duplex oligo concentration was adjusted to 200 nM. All other cleavage assays were performed as for multiple-turnover conditions, but the reactions were performed individually. In all assays, parallel control reactions were performed with NEIL1 alone to correct for the contribution of NEIL1 to the product formation. The products were separated by denaturing gel electrophoresis in 20% polyacrylamide, 7 M urea, and 1× Tris/borate buffer containing 89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8.4 (TBE). The radioactivity was quantitated using a PhosphoImager and Image Quant software. The Sigma Plot software was used for data analysis.

Electrophoretic Mobility Shift Analysis (EMSA). The affinity of OGG1 and NEIL1 for duplex oligos with lesions was quantitated by EMSA. Duplex oligos of identical sequence but distinct internal lesions in the 5′-^{32}P-labeled strand were incubated in a buffer (*7*) containing 20 mM Tris-HCl (pH 7.5), 100 nM NaCl, 1.0 mM EDTA, 1.5 μg bovine serm albumin, and 15% glycerol with different amounts of enzyme for various times at 30 °C and then electrophoresed in either TBE or Tris−glycine (pH 8.3) in 10% native polyacrylamide. The radioactivity in the separated bands was quantitated as before. Only one complex was observed for both NEIL1 (1−5 nM) and OGG1 (5−25 nM). We assumed equimolar binding and calculated the dissociation constant (K_d) from the binding isotherms by using the equation $D_b = 1/(1 + K_d[E])$, where D_b is the fraction of DNA-bound enzyme and E is its initial concentration (*23*). Sigma Plot software was used for the least-squares fit to calculate K_d and the standard deviation.

Cell Culture, Protein Extraction, and Quantitative Immunoblot Analysis. HeLa cells were grown in DMEM (Gibco, BRL) containing 10% fetal bovine serum and antibiotics (Gibco, BRL) at 37 °C in an incubator with 5% CO_2. For nuclear extract preparation, cells were harvested by gentle trypsinization and centrifugation and washed twice with ice-cold phosphate-buffered saline, and the nuclear extract was prepared as described before (*24*).

HeLa nuclear extracts and varying amounts of recombinant OGG1 or NEIL1 were denatured by boiling for 5 min in sample buffer and subjected to SDS−PAGE. The separated proteins were transferred to a nitrocellulose membrane, which was then incubated for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline before exposure to affinity-purified polyclonal antibodies raised in rabbit against human OGG1 and NEIL1 and subsequently with the secondary antibodies coupled to horseradish peroxidase. The bands were detected by chemiluminescence (ECL, Amersham Biosciences). The intensity of each band was quantitated by densitometry (Molecular Dynamics). The amounts of OGG1 and NEIL1 in HeLa nuclear extracts were determined by regression analysis from standard curves generated with purified recombinant OGG1 and NEIL1.

RESULTS

NEIL1 Stimulates Glycosylase Activity of OGG1. To determine the effect of NEIL1 on OGG1-catalyzed 8-oxoG excision, a constant amount of OGG1 (5 nM) was incubated with an 8-oxoG-containing oligo duplex in the presence of varying amounts of NEIL1 (2.5−50 nM) for 10 min at 37 °C. As shown in Figure 1, a NEIL1 dose-dependent increase in 8-oxoG excision (as measured by DNA strand incision) activity was observed until a plateau was reached with about 4-fold increase in OGG1 activity at an OGG1:NEIL1 molar ratio of 1:5. However, the stimulation of OGG1 by NEIL1 was significant even at the equimolar level of these enzymes. The NEIL1-mediated increase in the 8-oxoG excision activity of OGG1 observed here is not due to a background activity that may arise due to the low 8-oxoG release activity of NEIL1. This was confirmed directly by measuring incision at the 8-oxoG site by NEIL1 alone (Figure 1C). The NEIL1-mediated strand incision of 8-oxoG was less than 15% of total incision, consistent with previous reports (*16*, *19*).

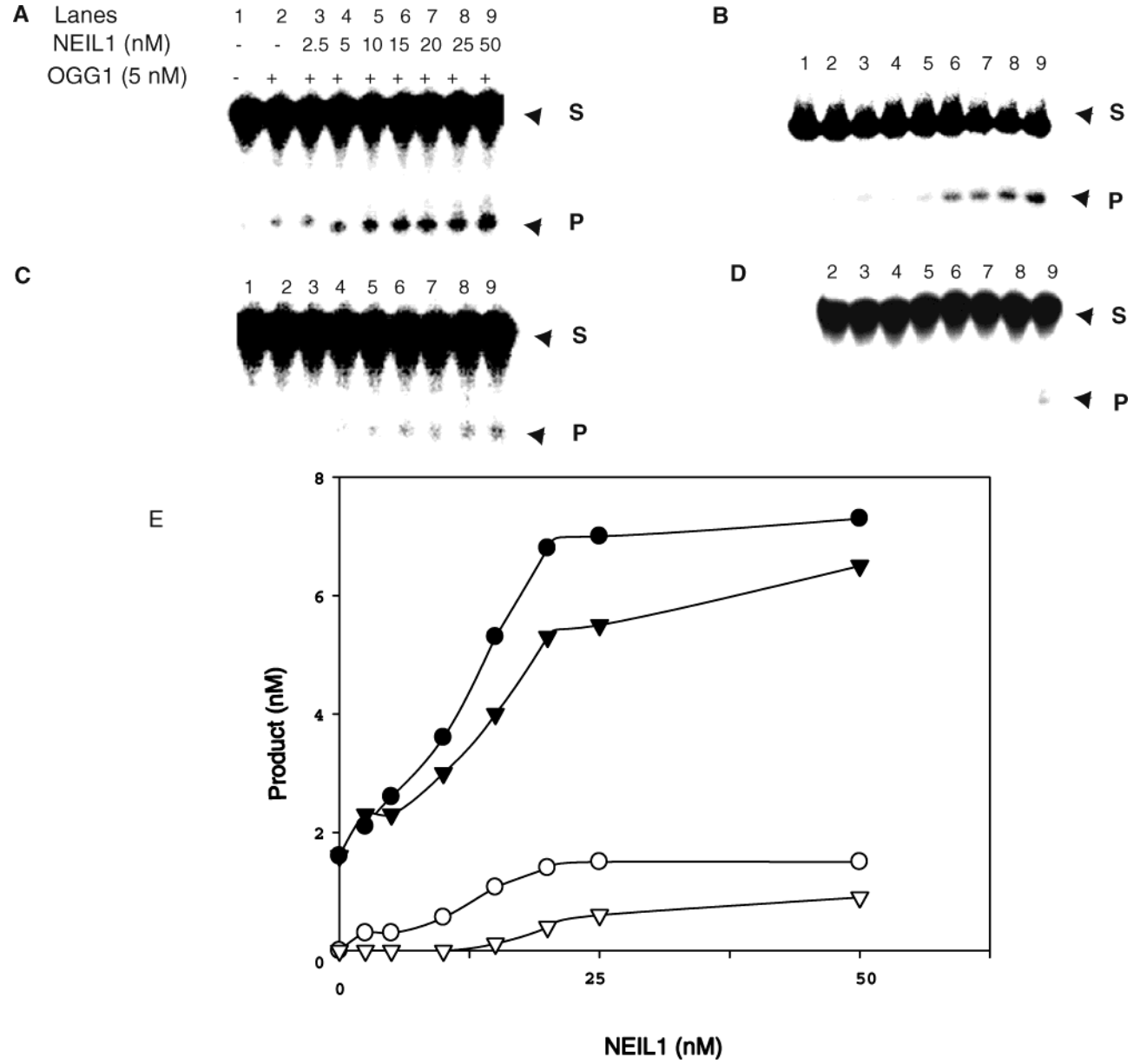


FIGURE 1: Stimulation of OGG1 glycosylase activity by NEIL1. OGG1 (5 nM) was incubated in the presence or absence of increasing concentrations of wild-type (A) or mutant NEIL1 (K53L) (B) with 5′-^{32}P-labeled 8-oxoG·C oligo (200 nM) for 10 min at 37 °C. Reactions with WT (C) or the K53L mutant (D). Concentrations of NEIL1 (lanes 1−9) were used in (B−D) as in (A). Kinetics of 8-oxoG excision after correcting for the NEIL1-mediated reaction (E). Key: S, substrate; P, product; (●) OGG1 with wild-type NEIL1; (▼) OGG1 with K53L NEIL1; (○) wild-type NEIL1 alone; (▽) K53L NEIL1 alone.

To determine whether the catalytic activity of NEIL1 is required for increasing the 8-oxoG excision activity of OGG1, a mutant of NEIL1 (K53L) deficient in DNA glycosylase activity was used at molar ratios similar to that used with WT NEIL1. The mutant NEIL1 also stimulated OGG1 activity but at a lower level with the same molar ratio (Figure 1B). Parallel reactions performed with the mutant NEIL1 alone showed barely detectable strand incision at the 8-oxoG site (Figure 1D).

OGG1 Does Not Influence NEIL1 Activity. To further confirm that the observed stimulation of 8-oxoG excision was not due to stimulation of NEIL1 by OGG1, we carried out the reciprocal experiment by analyzing the effect of OGG1 on NEIL1-catalyzed excision of 5-OHU and 8-oxoG using oligos of identical sequence except for the lesion. OGG1 is inactive with the 5-OHU substrate, which is an excellent substrate of NEIL1 (*16*). We used WT OGG1 for experiments with the 5-OHU-containing oligo and also a catalytically inactive OGG1 mutant (K249Q) in which the active site Lys-249 was substituted with Gln (*11*, *25*). It is evident that OGG1 did not influence the NEIL1 activity on either substrate (Figure 2) and thus confirms our conclusion that NEIL1 stimulates OGG1 and not vice versa.

Kinetics of Glycosylase Activity under Multiple-Turnover Condition. We investigated the effect of NEIL1 on OGG1 under the condition of multiple turnover with substrate [S] significantly in excess over the enzyme [E]. The data were fitted to the equation (*26*, *27*):

$$[P]_t = A_0\{1 - \exp(-k_{obs}t)\} + vt$$

where A_0 is the amplitude of the exponential phase, k_{obs} is the observed first-order rate constant, and v is the rate in the linear phase. Since the kinetic behavior of OGG1

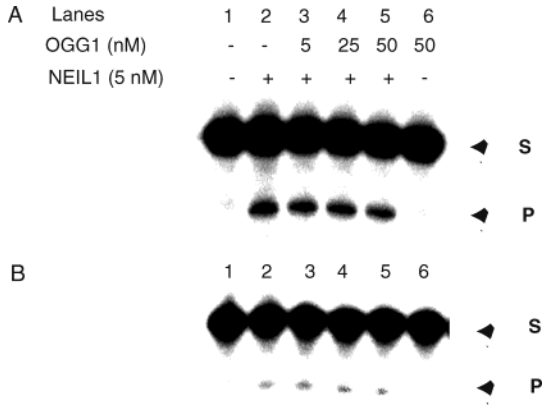
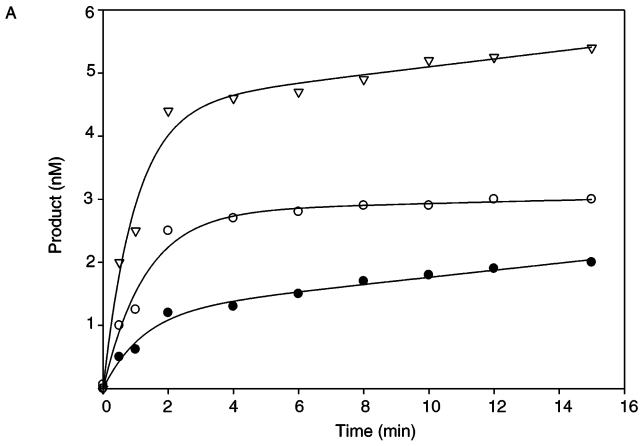


FIGURE 2: Effect of OGG1 on NEIL1 activity. Recombinant NEIL1 (5 nM) was incubated at 37 °C for 5 min with 200 nM 5-OHU·G oligo (A) or 8-oxoG·C (B) with or without varying amounts of wild-type (A) and (K249Q) OGG1 (B) as indicated. Other details are as in Figure 1 and in Experimental Procedures.



B

OGG1 concentration (nM)	Amplitude of burst	Active enzyme (%)
2.5	1.2	48
5.0	2.8	56
10.0	4.5	45

FIGURE 3: Determination of the active fraction in OGG1. OGG1 at 2.5 nM (●), 5 nM (○) or 10 nM (▽) was incubated with 200 nM 8-oxoG·C oligo at 37 °C, and product formation was measured at the indicated time points as described in Experimental Procedures (A). The fraction of active enzyme was estimated from the intercept after extrapolating the steady-state linear portion after the initial burst. The results are presented as a percentage of purified OGG1 (B).

(*7*, *28*) is similar to that of other DNA glycosylases (*26*, *27*), we determined the rate constants after calculating the fraction of active molecules in our purified enzyme preparations by following a scheme for the general DNA glycosylase/AP lyase-type enzyme initially developed (*29*) and later applied to other DNA glycosylases including OGG1 (*26*−*28*) (Figure 3). OGG1 in the absence of NEIL1 exhibited an initial high reaction rate (up to 2 min), after which the rate dropped drastically (Figure 4). The amount of cleaved 8-oxoG during the initial burst is equivalent to the amount of active enzyme present in the reaction. However, after the initial turnover, the product formation reached a plateau. These results strongly suggest very low turnover of the enzyme as was already observed. The burst kinetics indicate that a step after the base release, probably dissociation from the product AP site, is much slower than the base release as was also reported

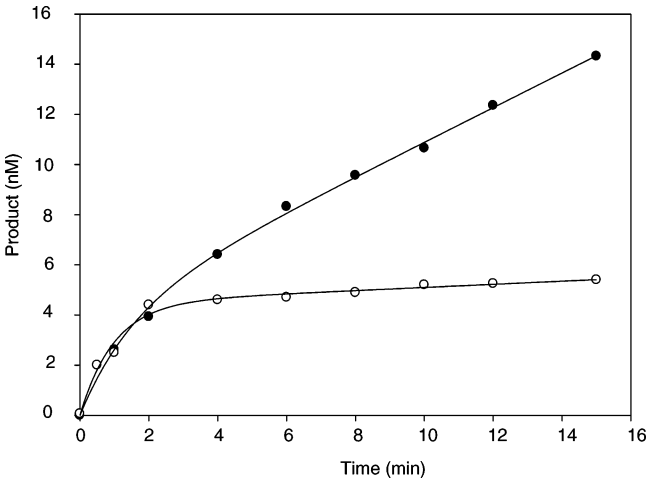


FIGURE 4: Kinetics of OGG1 under multiple-turnover conditions. OGG1 (5 nM) was incubated at 37 °C with 200 nM 8-oxoG·C oligo with or without NEIL1 (25 nM), and 8-oxoG excision was measured as described in Experimental Procedures. Key: (○) OGG1 alone; (●) OGG1 with NEIL1. A representative plot from three independent experiments is shown.

Table 1: Rate Constants of OGG1 under Various Assay Conditions[a]

	multiple turnover		single turnover	
	pre-steady-state base release	steady-state base release	base release	AP lyase
OGG1	1.7 ± 0.28	0.08 ± 0.01	2.2 ± 0.16	0.1 ± 0.01
OGG1 + NEIL1	1.4 ± 0.3	0.68 ± 0.03	1.8 ± 0.2	NA[b]

[a] Rate constants per minute for multiple- and single-turnover conditions were calculated from Figures 4 and 5, respectively. [b] Not applicable.

previously (*7*). In contrast, NEIL1, like APE1 examined earlier, enhanced the rate of the AP site release step without affecting the rate of base excision. The 9-fold higher rate of steady-state reaction without influencing the pre-steady-state rate constant of OGG1 in the presence of NEIL1 (Table 1) suggests that NEIL1 enhances turnover of OGG1.

Lack of Stimulation of OGG1 Activity by NEIL1 under Single-Turnover Condition. Analysis of OGG1 kinetics during steady-state reaction could not be performed accurately because of product inhibition. We therefore measured OGG1 activity under single-turnover condition in which the amount of product is a measure of the *N*-glycosyl bond cleavage and is not affected by the rate of product release. The single-turnover condition was imposed using [E] ≫ [S], and the reaction follows first-order kinetics for product formation: $[P]_t = A_0\{1 - \exp(-k_{obs}t)\}$, where [P] is the product concentration at the time (t). To make sure that the enzyme:substrate ratios that we used here are appropriate for examining pre-steady-state kinetics, we performed initial experiments with varying concentrations of the active enzyme. The enzyme concentration that gives the maximal rate was chosen. Under these conditions, doubling the concentrations of enzyme and DNA did not affect the rate. Single-turnover profile (Figure 5) and rate constants calculated therefrom show no stimulatory effect of NEIL1 on the glycosylase activity of OGG1. This indicates that NEIL1 does not affect the catalysis of the 8-oxoG excision reaction. These experiments were carried out at a molar ratio of OGG1 to NEIL1 of 1:2 rather than 1:5 used in previous multiple-

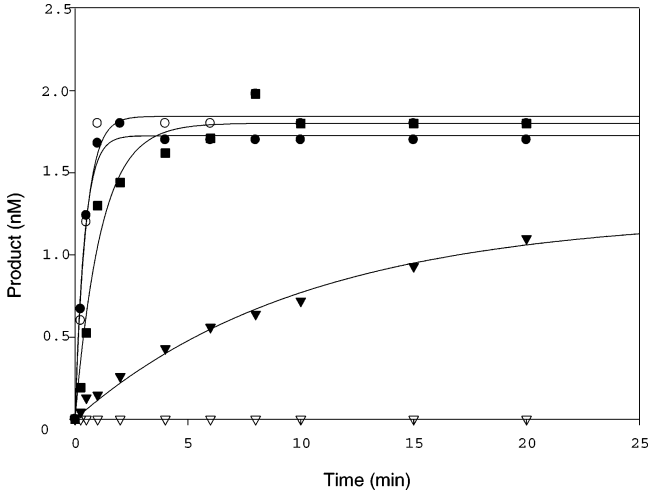


FIGURE 5: Single-turnover kinetics of OGG1. OGG1 (10 nM) was incubated at 37 °C with 2 nM 8-oxoG·C oligo with or without NEIL1 (20 nM). Other conditions are as in Figure 3 and described in Experimental Procedures. Key: (●) glycosylase activity of OGG1; (○) glycosylase activity of OGG1 with NEIL1; (▼) AP lyase activity of OGG1; (▽) AP lyase activity of OGG1 with NEIL1; (■) AP lyase activity of NEIL1 with OGG1. A representative plot from three independent experiments is shown.

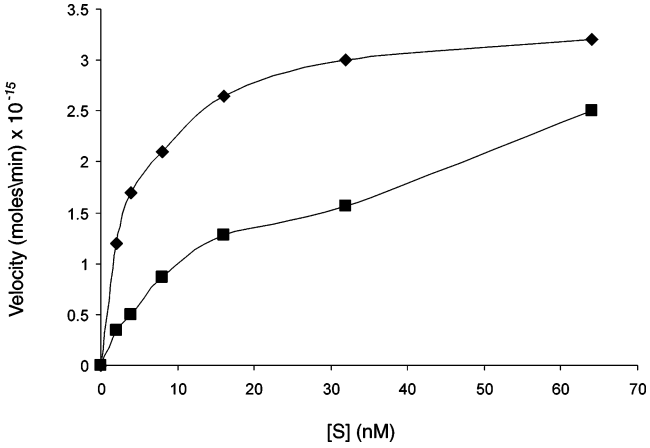


FIGURE 6: OGG1 activity as a function of substrate concentration. OGG1 (2.5 nM) was incubated in the presence or absence of NEIL1 (12.5 nM) with increasing concentrations of 8-oxoG·C oligo (2−64 nM) for 5 min at 37 °C, and the product was analyzed as before. Key: (■) OGG1 alone; (◆) OGG1 with NEIL1. A representative plot from three independent experiments is shown.

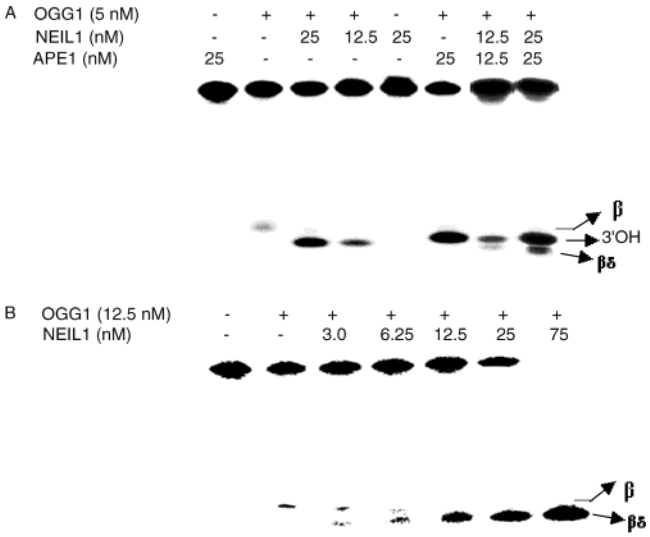


FIGURE 7: Effect of NEIL1 on 3′-termini generated by OGG1. OGG1 was incubated in the presence or absence of various concentrations of NEIL1 or APE1 with 5 nM 8-oxoG·C oligo (A) or 200 nM AP·C (B) oligo at 37 °C for 10 min. 1 mM $MgCl_2$ was present in reactions containing APE1. After the reactions were terminated with SDS (0.5%) and glycerol (5%), the products were analyzed in 20% polyacrylamide/7 M urea. The β and $\beta\delta$ elimination and 3′-OH containing products are indicated.

turnover experiments for technical reasons. We observed inhibition of glycosylase activity of OGG1 with limited substrate concentration when excess NEIL1 was present in the reaction mixture, presumably because of nonspecific binding of NEIL1 to the 8-oxoG·C substrate. Complete abrogation of the AP lyase activity of OGG1 was observed, consistent with the results obtained in the 3′ end analysis as described below. We conclude that NEIL1 does not enhance the rate of base cleavage reaction of OGG1 (Table 1) and thus does not affect the catalysis step of OGG1. The time required to complete the first cycle of the burst curve (approximately 2 min; Figure 4) is similar to the time required to attain the saturation (2 min) in single-turnover kinetic analysis of OGG1 alone (Figure 5). A similar value of the burst time was also obtained in our previous studies (*7*).

Effect of NEIL1 on the Kinetics of OGG1. We showed earlier that OGG1 does not follow Michaelis−Menten kinetics (*7*). We predicted that OGG1 in the presence of NEIL1 would follow the conventional kinetics. Figure 6 confirms this prediction. As expected, OGG1 alone had low turnover, and the reaction rate continued to increase with substrate concentration. However, NEIL1 like APE1 enhanced turnover of OGG1, resulting in a typical Michaelis−Menten curve.

Analysis of the 3′ Incision Product Generated by OGG1 with and without NEIL1. We investigated the nature of 3′ DNA termini generated by OGG1 in the presence of NEIL1. The processing of the 3′ end determines the next step in the repair process. Figure 7A shows that addition of NEIL1 to the OGG1 reaction mixture exclusively produced the $\beta\delta$-elimination product with the 3′-phosphate terminus. Parallel reactions performed with human APE1 showed the incision product of 3′-OH termini, as expected. Interestingly, the presence of all three enzymes together showed the formation of both 3′-OH and 3′-phosphate termini, generated by APE1 and NEIL1, respectively. Thus, the presence of an equimolar amount of APE1 did not affect NEIL1-generated 3′-phosphate termini. Lack of any cleavage of the oligo with APE1 or NEIL1 alone confirms the lack of AP site contamination in the oligo or Fpg contamination in purified NEIL1. These results are further supported by experiments with AP site-containing oligo, where the AP lyase activity of OGG1 was suppressed due to the strong AP lyase activity of NEIL1 (Figure 7B).

Analysis of the OGG1-Trapped Complex and OGG1−THF·C Complex in the Presence of NEIL1. All AP lyases carrying out either β- or $\beta\delta$-elimination reaction form a transient Schiff base adduct with the deoxyribose at the AP site which could then be converted into a stable "trapped complex" by reduction with $NaBH_4$ or $NaCNBH_3$ (*4*). DNA glycosylases can be distinguished from one another in a mixture by the characteristic mobility of their trapped complexes in SDS/PAGE (*30*). To test whether the 3′-

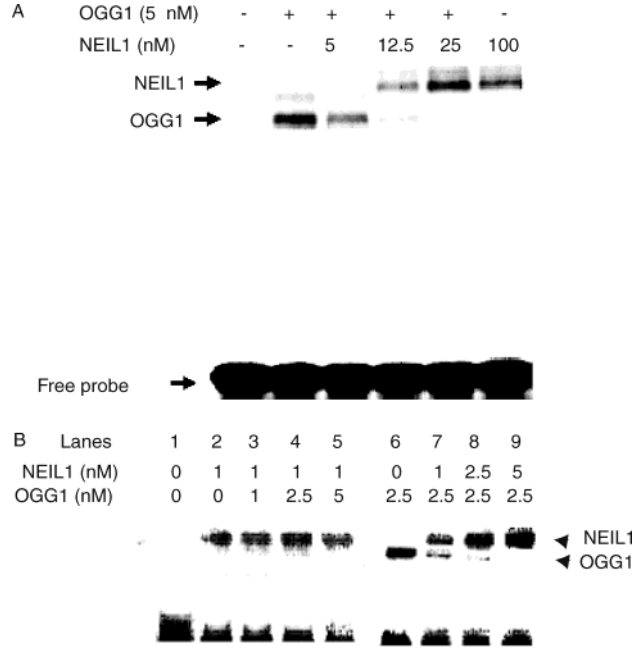


FIGURE 8: Effect of NEIL1 on formation of the trapped complex and THF·C binding of OGG1. (A) 5′-^{32}P-labeled 8-oxoG·C oligo (50 nM) was incubated with OGG1 and varying concentrations of NEIL1 in the presence of 50 mM $NaBH_4$ at 37 °C for 30 min and analyzed by SDS/PAGE (10% polyacrylamide). (B) 5′-^{32}P-labeled THF·C-containing duplex oligo (0.5 nM) was incubated at 30 °C for 10 min at either NEIL1 (lanes 2−5) or OGG1 (lanes 6−9). The DNA−protein complex was then challenged with OGG1 (lanes 2−5) or NEIL1 (6−9) by further incubation for an additional 10 min. The products were separated on 10% native polyacrylamide in Tris−glycine buffer.

phosphate termini generated by NEIL1 were due to δ-elimination reaction with the OGG1 β-elimination product or due to direct $\beta\delta$-elimination reaction of the AP site, we examined the effect of NEIL1 on the level of the OGG1-trapped complex. Because of the smaller size of OGG1, its complex should migrate faster than the NEIL1-trapped complex in SDS/PAGE, formed with the same oligo. A dose-dependent decrease in the OGG1-trapped complex with the increasing amount of NEIL1 in the trapping reaction (Figure 8A) indicates that NEIL1 competed for the OGG1-bound AP site. We confirmed higher affinity of NEIL1 over OGG1 for the nonhydrolyzable AP site analogue, tetrahydrofuran (THF), by carrying out EMSA as described in Experimental Procedures. The dissociation constants derived from the EMSA experiments showed a higher affinity of NEIL1 (with a K_d of 2.5 ± 0.5 nM) than OGG1 (7 ± 1.0 nM) for AP site analogue.

We then carried out competition experiment to test directly whether NEIL1 competes for THF with OGG1. Figure 8B shows that NEIL1 could displace OGG1 in the THF oligo-bound complex, as indicated by formation of the slower migrating band with increasing amounts of NEIL1. In contrast, OGG1 could not displace NEIL1 in a reciprocal experiment. We thus conclude that NEIL1 displaces OGG1 from the AP site due to higher affinity.

Affinity of OGG1 for DNA 3′-Phosphate. We had earlier measured the affinity of OGG1 for its substrate and products (*7*). In the present study, we examined the affinity of OGG1 for the DNA 3′-phosphate terminus, the product of the NEIL1 reaction. Lack of stable interaction of OGG1 with 3′-

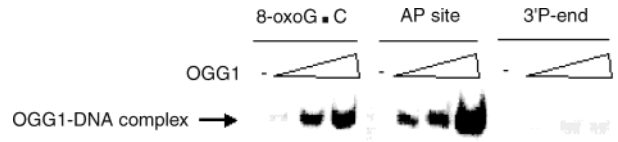


FIGURE 9: EMSA of OGG1 with DNA containing 3′-phosphate. K249Q OGG1 (0.25, 0.5, and 1.0 nM) was incubated with 2 nM 5′-^{32}P-labeled oligo with 3′-phosphate at the strand break. 8-oxoG·C and AP·C containing oligos of identical sequence were used as controls. Other details are described in Experimental Procedures.

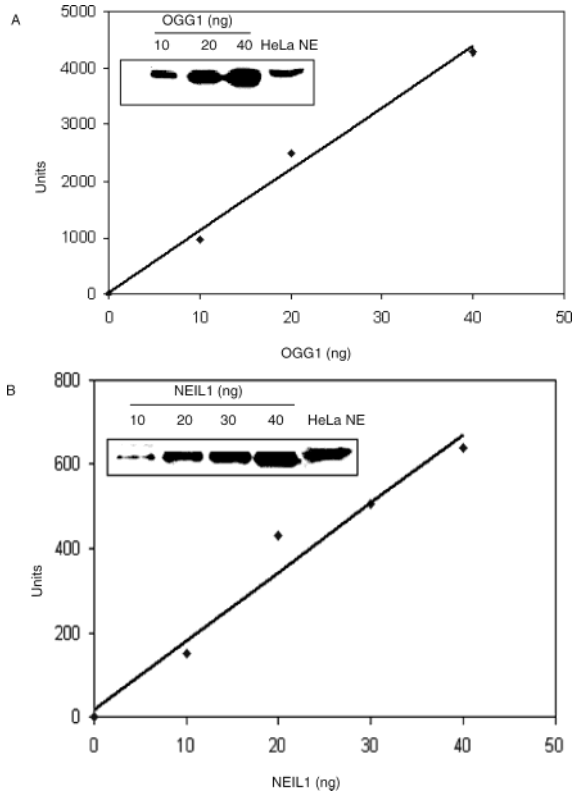


FIGURE 10: Estimation of relative amounts of OGG1 and NEIL1. HeLa nuclear extract (HeLa NE) obtained from 1×10^7 cells and indicated amounts of recombinant OGG1 (A) and NEIL1 (B) were subjected to 10% SDS−PAGE. Standard curves were derived from Western analysis (insets) as described in Experimental Procedures.

phosphate-containing oligo but with the 8-oxoG·C and AP site-containing oligo duplexes of the same sequence indicates that OGG1 did not reassociate with the product of NEIL1 (Figure 9).

NEIL1 and OGG1 Levels in the HeLa Nuclear Extract. To assess the physiological relevance of stimulation of OGG1 activity by NEIL1, we determined the relative abundance of these two enzymes in the nuclear extract prepared from log-phase HeLa cells by quantitative immunoblotting (Figure 10). On the basis of the molecular masses of NEIL1 (44 kDa) and OGG1 (38 kDa), we have calculated that the number of NEIL1 molecules per cell was about twice that of OGG1 (Table 2). This suggests that NEIL1 activation of OGG1 could be physiologically significant.

DISCUSSION

Many mammalian DNA glycosylases have strong affinity for AP sites and turn over poorly after excising base lesions from DNA. The stimulation of these enzymes by APE1 was first observed with Udg (*12*). Subsequent structural elucida-

Table 2: Abundance of OGG1 and NEIL1 in HeLa Nuclear Extract[a]

	OGG1	NEIL1
amount (ng)/75 μg of nuclear extract	10 ± 1.6	24 ± 1.4
no. of enzyme molecules per cell	16000 ± 2560	32700 ± 4580

[a] The number of enzyme molecules per cell was calculated from Figure 10. The data indicate the mean ± SD from three independent experiments.

tion of the product-bound APE1 by X-ray crystallography led to the hands-off (or baton-passing) model in BER (*9*, *10*). This model postulates that the repair steps in BER are coordinated by product binding of the enzyme. Thus a DNA glycosylase after excising the lesion base remains bound to its own product and hands it over to the APE, the next enzyme in the repair pathway. This sequestration would prevent undesirable side reaction of the product (*9*). The affinity of many DNA glycosylases for their products is manifested in their non-Michaelis−Menten kinetics. In the case of OGG1 and other DNA glycosylases stimulated by APE1, this handover does not involve their stable interaction (*31*). However, other BER-associated proteins, e.g., XPG, YB-1, and XRCC1, were shown to stably interact and stimulate other DNA glycosylases such as NTH1 and OGG1 (*32*−*34*).

In the present study, we have made a rather unexpected observation that OGG1 could be stimulated not only by APE1 but also by another DNA glycosylase, NEIL1, which by itself has very weak 8-oxoG excision activity. We have eliminated the possibility that this weak activity contributes significantly to enhanced 8-oxoG excision by using several approaches. First, we examined the effect of OGG1 and NEIL1 together on excision of 5-OHU from an oligo duplex of the same sequence as for 8-oxoG. OGG1 does not recognize 5-OHU as a substrate. We observed no enhancement of 5-OHU excision by NEIL1 in the presence of OGG1. Second, we showed that a catalytically inactive NEIL1 K53L mutant, which lacks both *N*-glycosylase (*22*) and AP lyase activity (T. K. Hazra, unpublished experiment) also stimulated OGG1 turnover although to a smaller extent. The diminished effect of the mutant could be due to significant alteration in its tertiary structure which decreases its affinity for the AP site. Finally, far Western and co-immunoprecipitation studies indicated a lack of direct interaction between NEIL1 and OGG1 (data not shown). Taken together, these results clearly show that NEIL1 enhances OGG1 turnover by displacing it from the bound AP site product. Thus NEIL1, and possibly NEIL2, may have two distinct roles in repair of oxidatively damaged bases. They act directly in excision of these bases and could also act on the AP site generated due to removal of these bases by other DNA glycosylases.

NEIL1 may influence any of the following steps of OGG1 activity: (i) base release (*32*), (ii) AP lyase activity (*32*), (iii) turnover of the enzyme (*7*, *8*, *33*), or (iv) affinity of the enzyme for its substrate (*35*). To identify the step(s) affected by NEIL1, we performed single-turnover and multiple-turnover kinetics. Under the condition of multiple turnover in the presence or absence of NEIL1, the rate of enzymatic reaction during the initial burst was the same; subsequently, a slower rate of product formation was observed with OGG1 alone, in contrast to the steady rate in the presence of NEIL1. Consistent with these results, we observed no difference in the rate of base release in the presence or absence of NEIL1 under conditions where [OGG1] ≫ [S]. These results are similar to those we had reported earlier on OGG1 stimulation by APE1 (*7*). Similar stimulation of hNTH1 by APE1 was later reported (*33*). When checked for the AP lyase activities of OGG1 in the presence of NEIL1, all enzymatic cleavage products were found to be of the $\beta\delta$-elimination type. When we compared the rate of base release and AP lyase activities, the rate of base release by OGG1 (2.4 ± 0.18) is approximately 2.5 times faster than the subsequent rate of AP site cleavage by NEIL1 (0.87 ± 0.12) in a reaction containing both OGG1 and NEIL1 (Figure 5). This suggests that in the presence of NEIL1 all AP sites produced by OGG1 are taken over by NEIL1 and cleaved to produce 3′-phosphate termini. OGG1 removed all bases within 2 min, and it took 6 min for NEIL1 to cleave all of the OGG1-generated AP sites. The delay in the rate of AP site processing is in agreement with the rate of enzymatic reactions for these two steps. In contrast, OGG1-mediated base release and AP lyase steps are well separated due to the weak AP lyase activity of OGG1 (*36*, *37*), and the time required to cleave all of the AP sites produced under single-turnover condition was more than 20 min, and the rate of AP lyase activity was 25 times less than the base release step (Table 1). Although the rate constant calculated for AP lyase activity is close to the values reported earlier, the rate constant for base release is significantly lower than the one reported before (*28*). The observed difference could be due to the difference in the sequence context of the substrate lesion which was shown to have a marked influence on the base excision activity of yeast OGG2 (*38*, *39*). We ruled out the possibility that this difference could be due to incomplete cleavage of AP sites by piperidine because similar results were obtained with piperidine vs 0.1 N NaOH. However, we did see a higher rate of base excision in Tris vs HEPES buffer routinely used in our study. The strand cleavage products with OGG1 were exclusively of β-elimination type, as expected. In contrast, addition of NEIL1 caused generation of 3′-P ends characteristic of NEIL1 (Figure 7) for which there are two possible mechanisms: (i) NEIL1 first stimulates AP lyase activity of OGG1, and thus subsequent cleavage of the β-elimination product by NEIL1 leads to the observed 3′-phosphate product. (ii) Alternatively, NEIL1 displaces OGG1 bound to the AP site after the *N*-glycosyl bond cleavage and cleaves the AP site to produce the $\beta\delta$-product. Because neither the turnover kinetics nor 3′-end analysis could distinguish between these possibilities, we analyzed the OGG1 trapped complex formed in the presence or absence of NEIL1. A decrease in the OGG1-trapped complex with increasing concentrations of NEIL1 (Figure 8A) indicated that NEIL1 overrides OGG1 AP lyase reaction because of its higher affinity for the AP site and stronger AP lyase activity (Figures 8B and 7B). These results are similar to the previous observation about APE1 stimulation of OGG1. Thus NEIL1 generates the 3′ end termini directly from the cleavage of AP sites produced by OGG1.

[2] E. B. Jackson, Jr., and T. Izumi, unpublished results.

Unlike in the case of other proteins, NEIL1 activation of OGG1 could not be simply explained by the hands-off model or the result of stable interaction as was observed between NTH1 and XPG (*35*). However, despite an apparent absence of a simple rationale for collaboration of NEIL1 and OGG1 in carrying out repair of oxidized base lesions particularly when APE1 is available, it is reasonable to suggest that NEIL1 could enhance OGG1 turnover in vivo for several reasons. First, OGG1 and NEIL1 at least in log-phase HeLa cells have comparable abundance (Table 2). In contrast to the cell cycle-independent level of OGG1 (*40*), NEIL1 is activated during the S phase (*16*). Because NEIL1 may have significantly higher abundance than OGG1 in S-phase cells, it could activate OGG1 in vivo. Second, while it is possible that APE1 with much higher cellular level is more likely to be responsible for enhancing OGG1 turnover, the higher cellular abundance of APE1 may be misleading (*41*). A significant fraction of APE1 is present in the cytosol and translocates to the nucleus in response to external stimuli including oxidative stress[2] (*42*). Further, although the APE1 level may significantly exceed that of NEIL1, because of its multifunctional nature (*43*−*46*), only a fraction of this enzyme may be involved in BER. In contrast, the NEIL1 molecules should be fully engaged in repair in the absence of any other known function and could have a secondary role in stimulating OGG1 turnover in vivo.

Finally, NEIL1-mediated AP site cleavage raises the question of how the 3′-phosphates are removed to produce the 3′-hydroxyl terminus necessary for subsequent repair synthesis by DNA polymerases. The mammalian APE1, the key enzyme to remove the β-elimination product of human NTH1 and OGG1 (*33*, *47*), needed for repair synthesis by Pol β action, has extremely weak 3′-phosphatase activity (*43*, *48*, *49*). We have now shown that polynucleotide kinase, a DNA 5′-kinase/3′-phosphatase, abundant in mammalian cells, but absent in *E. coli*, is responsible for removing the 3′-phosphate (*50*). Thus the discovery of APE-independent NEIL1/PNK-dependent repair of oxidative base damage indicates the presence of an alternative repair process in human cells. Our observation of NEIL1 enhancement of 8-oxoG excision by OGG1 and subsequent repair via this pathway (*50*) underscores the multiple ways of repairing oxidative base damage, commensurate with their contribution to genotoxicity.

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