Repair of oxidatively damaged bases in the genome via the base excision repair pathway is initiated with excision of these lesions by DNA glycosylases with broad substrate range. The newly discovered human DNA glycosylases, NEIL1 and NEIL2, are distinct in structural features and reaction mechanism from the previously characterized NTH1 and OGG1 but act on many of the same substrates. However, NEIL2 shows a unique preference for excising lesions from a DNA bubble, whereas NTH1 and OGG1 are only active with duplex DNA. NEIL1 also excises efficiently 5-hydroxyuracil, an oxidation product of cytosine, from the bubble and single-stranded DNA but does not have strong activity toward 8-oxoguanine in the bubble. The dichotomy in the activity of NEILs versus NTH1/OGG1 for bubble versus duplex DNA substrates is consistent with higher affinity of the NEILs for the bubble structures of both damaged and undamaged DNA relative to duplex structure. These observations suggest that the NEILs are functionally distinct from OGG1/NTH1 in vivo. OGG1/NTH1-independent repair of oxidized bases in the transcribed sequences supports the possibility that NEILs are preferentially involved in repair of lesions in DNA bubbles generated during transcription and/or replication.

Reactive oxygen species are continuously generated as by-products of respiration and are also induced during the inflammatory response and other pathological processes (1, 2). These compounds and radicals are genotoxic and in addition to DNA strand breaks induce a plethora of base lesions, many of which are mutagenic and/or toxic (3). Most of the damaged bases are repaired via the base excision repair pathway that is initiated with their excision by DNA glycosylases with broad substrate range (4). Until recently, only two DNA glycosylases have been identified and characterized in mammalian cells as being responsible for repairing oxidatively damaged bases, 8-oxoguanine-DNA glycosylase (OGG1) and endonuclease three homolog 1 (NTH1). OGG1 and NTH1 have been shown to have significant sequence homology. NTH1 shares many of these substrates including thymine glycol, and 5-hydroxyuracil (5-OHU) as substrates, whereas NEILs show a unique preference for excising lesions from DNA bubble structures. In contrast, NEIL2 excises 5-OHU and other oxidized derivatives of cytosine (17–18); NTH1 shares many of these substrates including 5-OHU with NEIL1 and NEIL2 (17, 18, 25). Surprisingly, except for few conserved motifs, NEIL1 and NEIL2 show no significant sequence homology.

Here we report an unusual activity of NEIL1 and NEIL2 in excising lesions from DNA bubble structures. In contrast, neither OGG1 nor NTH1 shows any base excision activity for lesions present in a bubble or single-stranded (ss) DNA. This novel structural preference of the NEILs raises the possibility that these enzymes are involved in a distinct function in vivo for the repair of oxidized bases.

**EXPERIMENTAL PROCEDURES**

Oligonucleotide Substrates—Two 51-mer oligos containing either 5-OHU or 8-oxoG at position 26 from the 5′-end (shown in Table I) were purchased from Midland Certified Reagent. The undamaged control oligo contained C at position 26. The sequences of complementary

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† To whom correspondence should be addressed: Sealy Center for Molecular Science and Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77555.

‡ The abbreviations used are: OGG1, 8-oxoguanine-DNA glycosylase 1; AP, abasic; 8-oxoG, 7,8-dihydro-8-oxoguanine; EMSA, electrophoretic mobility shift analysis; 5-OHU, 5-hydroxyuracil; Nei, endonuclease VIII; NER, nucleotide excision repair; NEIL, Nei like; nt, nucleotides; NTH1, endonuclease III homolog 1; ss, single-stranded; TCR, transcription-coupled repair; WT, wild type; oligo, oligonucleotides.

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**Hong Dou, Sankar Mitra, and Tapas K. Hazra‡**

*From the Sealy Center for Molecular Science and Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77555*
oligos, purchased from Invitrogen, containing G or C opposite the lesion or containing sequences for producing bubble structures containing 5, 11, and 19 unpaired bases, and named B5, B11 and B19 respectively, are also shown in Table I. Two hundred fifty pmol each of the lesion strand and a complementary strand were heated at 94 °C for 2 min in 50 μl of phosphate-buffered saline and then slowly cooled to room temperature. To produce ~32P-labeled substrates, the single-stranded oligos were labeled at the 5′ terminus with [γ-32P]ATP and polynucleotide kinase before annealing.

**Enzymes**—Recombinant, wild type (WT) NEIL1, NEIL2, and OGG1 were purified to homogeneity from *E. coli* (17, 18, 26). The K53L mutant of NEIL1, encoding a C-terminal His tag fusion polypeptide, was generated in the pRSET(B) expression vector. The K49R mutant of NEIL2, without a C-terminal His tag fusion polypeptide, was generated in the pRSET(B) expression vector. Both proteins were expressed in *E. coli* and the K49R mutant was inactive in assays for AP lyase activity in DNA glycosylases after lesion excision, was assayed by using duplex oligo containing 5-OHU with C in position 26 (Table I) with either 5-OHU-containing or a normal strand with various complementary strands. The rate of product formation was linear for both enzymes under these conditions. The radioactivity in the DNA bands was quantitated by PhosphoImager analysis as before. The dissociation constants of binding were calculated according to Taylor et al. (28).

**Incision Assay of DNA Glycosylases**—DNA strand cleavage, due to intrinsic AP lyase activity of DNA glycosylases after lesion excision, was assayed by using duplex oligo containing 5-OHU or 8-oxoG in the ~32P-labeled strand, after incubation with the DNA glycosylases at 37 °C for the indicated times in 10-μl reactions containing 40 μM Hepes-KOH (pH 7.5), 50 mM KCl, and 100 μM/ml bovine serum albumin. The reactions were stopped with 70% formamide, 30 mM NaOH, and the cleaved oligo products were then separated by denaturing gel electrophoresis in 20% polyacrylamide gel containing 7% urea, 90 mM Tris borate (pH 8.3), and 2 mM EDTA. For analysis of enzyme kinetics, we incubated 5-OHU-containing duplex oligos confirmed our prediction that B5 and B19 bubble oligos have similar base unpaired regions flanked by duplex segments.

**Preferential Excision of 5-OHU from a DNA Bubble Structure**—We compared the DNA glycosylase activity of NEIL1, NEIL2, and NTH1 in excising 5-OHU from duplex, single-stranded, and bubble oligos B5, B11, and B19 with 5, 11, and 19 unpaired bases, respectively. The lesion base was positioned in the middle of the bubble in all cases. We have confirmed the observation by Takao et al. (20), as shown in Fig. 3, that NEIL1 is active with ssDNA (Fig. 3A, lane 6), and we further showed that NEIL2 (Fig. 3A, lane 11) but not NTH1 (Fig. 3B, lane 5).
was similarly active with ssDNA. More remarkably, both NEIL1 and NEIL2 were highly active in excising 5-OHU when it was placed inside a bubble in B5, B11, and B19, in an otherwise duplex DNA (Fig. 3A, lanes 3–5 and 8–10). In contrast, NTH1 was completely inactive with the same substrate (Fig. 3B, lanes 3 and 4). This was expected, because NTH1 was also inactive with the ssDNA substrate (Fig. 3B, lane 5), as was shown before. NEIL1 is generally more active in base excision from duplex DNA than NEIL2 (17, 18). However, although both NEIL1 and NEIL2 had comparable activity with the bubble substrates, NEIL2 showed almost 4-fold higher excision activity when 5-OHU was present in an 11- or 19-nt bubble than in a duplex.

We determined the kinetic parameters of NEIL1 and NEIL2 with oligos containing 5-OHU either paired with G or present in the B11 bubble. The G/H18528 5-OHU pair should be generated in the genome in situ after oxidative deamination of C (31). Both NEIL1 and NEIL2 have higher catalytic specificity in excising 5-OHU from an 11-nt bubble than from a G/H18528 5-OHU pair in the duplex (Table II). Although both NEIL1 and NEIL2 prefer 5-OHU located in the bubble, NEIL1 has higher turnover and catalytic specificity than NEIL2. On the other hand, the preference of NEIL2 for the 11-nt bubble substrate relative to duplex DNA is higher (about 7-fold) than that of NEIL1 (about 3-fold).

Fig. 3. Activity assay of NEIL1, NEIL2, and NTH1 with substrates (500 nM) in different structures. Identical 5-OHU-containing oligo strands were used as is (5-OHU-ss) or annealed with a complementary strand containing G opposite 5-OHU (5-OHU/G) or with a noncomplementary strand to generate B5, B11, or B19 bubbles flanked by duplex sequences, as described under “Experimental Procedures.” A, relative activity of NEIL1 and NEIL2. B, activity of NTH1 (100 nM) was measured as in A. S, substrate; P, product.

Preferential Excision of 8-OxoG in a Bubble Structure—We showed earlier that with the duplex DNA NEIL1 but not NEIL2 was active in excising 8-oxoG from an 8-oxoG-C pair (17, 18). However, we have now observed that NEIL2 could excise 8-oxoG when it was present inside a bubble (Fig. 4, lane 6). Like NTH1, OGG1, the major 8-oxoG-excising enzyme in eukaryotic cells, was completely inactive in excising 8-oxoG from both a bubble structure or ssDNA (Fig. 4, lanes 9 and 10). Interestingly, NEIL1 had higher 8-oxoG excision activity when the lesion was present in the 8-oxoG-C duplex (lane 2) than in the bubble (lane 3).

Affinity of NEIL1 and NEIL2 for Bubble Conformation—The lower $K_m$ values of both NEIL1 and NEIL2 for the 5-OHU substrate in bubble relative to duplex DNA suggested that these enzymes have intrinsic affinity for the bubble structure.
We therefore analyzed their relative affinity for duplex versus bubble DNA by EMSA. Because the activity of NEIL1 and NEIL2 does not require a cofactor, we could not carry out EMSA with WT NEILs and substrate oligos. We therefore used inactive mutants of the enzymes with 5-OHU-containing oligos. We also examined the affinity of WT NEIL1 and NEIL2 with control oligos that are identical to the 5-OHU-containing oligos except for substitution of 5-OHU with C. Fig. 5 shows a representative analysis of WT NEIL1 and NEIL2 complexes with duplex and bubble-containing DNA with normal bases. The slower moving complex with bubble DNA appears to be due to the binding of two enzyme molecules which became more pronounced with increasing protein concentration (Fig. 6). It should be noted that NEIL1 and NEIL2 have no excision activity with normal bases, as expected (data not shown).

The affinity of WT NEIL1 and NEIL2 for normal DNA was calculated from the two-step binding reaction 1,

\[
\begin{align*}
K_{d1} & \quad D + P \rightleftharpoons DP_1 + P \rightleftharpoons DP_2 \\
\text{REACTION 1}
\end{align*}
\]

where \(D\) is free duplex or bubble oligo, and \(P\), NEIL1 or NEIL2, is present in significant excess over DNA. \(K_{d1}\) and \(K_{d2}\) are the equilibrium constants for primary and secondary binding.

Assuming the free duplex as the reference state, the partition function, \(Q\), for the binding reaction is given by Reaction 2 (28, 32).

\[
Q = |D| + K_{d1} \cdot |D| \cdot |P| + K_{d2} \cdot |D| \cdot |P|^2
\]

\[
\text{REACTION 2}
\]

With this definition, \(F\), the fraction of duplex bound in the primary mode (the first shifted band) is given by Reaction 3.

\[
F = \frac{K_{d1} \cdot |P|}{1 + K_{d1} \cdot |P| + K_{d2} \cdot |P|^2}
\]

\[
\text{REACTION 3}
\]

With two complexes and knowing the relative concentrations of these complexes and free DNA, the fraction of bound DNA was measured at various enzyme concentrations. \(K_{d1}\), i.e. \(1/K_{d1}\), could then be calculated from the binding isotherms by data fitting using the Sigma plot (representative plots are given in Fig. 6, B and C).

We calculated the apparent \(K_d\) values of interaction of WT NEIL1 and NEIL2 with normal duplex and B11 bubble oligos (Table III, top). We could not calculate the \(K_d\) values of these enzymes for single-stranded oligos because the complexes were not stable during EMSA. Interestingly, our initial EMSA results using the Tris borate buffer system were not reproducible.

On the other hand, the results obtained from gels containing Tris-glycine buffer were reliable and used in calculating the above constants. We carried out similar binding studies of K53L NEIL1 and K49R NEIL2 mutants with 5-OHU-containing oligos. Table III, bottom, summarizes these results. It is evident from these EMSA results that both WT and mutant NEILs have an inherent affinity for the bubble structures. Similar EMSA studies with NTH1 and OGG1 did not indicate the formation of stable complexes of these enzymes with single-stranded and 11-nt bubble oligos containing normal bases (data not shown).

DISCUSSION

Mammalian NEIL1 and NEIL2 share many oxidized DNA base lesions as substrates with OGG1 and NTH1, the other two previously characterized DNA glycosylases involved in repair of these lesions. However, unlike the latter, which belong to the Nth family, NEIL1 and NEIL2 belong to the distinct Fpg/Nei family of enzymes based on reaction chemistry. It is interesting that there is no significant homology between NEIL1 and NEIL2 (17, 18). Nevertheless, we show here that NEIL1 and NEIL2 share a common preference for an unusual DNA structure. Most DNA glycosylases of both bacterial and eukaryotic origin are active only with duplex DNA substrates. This is
expected because the complementary strand serves as a template for the repair of gaps generated in the lesion-containing strand after damage removal. Thus, neither NTH1 nor OGG1 showed detectable activity for excising 5-OHU or 8-oxoG from ssDNA. In contrast, NEIL1 and NEIL2 have significantly higher activity and catalytic specificity for 5-OHU located in a single-stranded sequence in a bubble than for those in duplex DNA. Furthermore, the activity of NEIL2 was much higher with the bubble substrate than with ssDNA. We used 5-OHU and 8-oxoG in oligos of the same sequence as common substrates for the DNA glycosylases in order to avoid any potential impact of the sequence context. We confirmed an earlier observation that NEIL1 is active with ssDNA (20), and we showed for the first time that NEIL2 has similar activity.

Our results indicate that NEIL1 had comparable activity with ssDNA and duplex DNA and with DNA containing 5–19-nt bubbles. In contrast, NEIL2 had much higher activity with the bubble DNA than with duplex or ssDNA. The preference of NEIL2 for the bubble substrate extends to repair of 8-oxoG (Fig. 3). Compared with a robust activity with the 8-oxoG-C pair in duplex DNA, OGG1 showed no activity when 8-oxoG was present in ssDNA or in an 11-nt bubble.

The complex substrate preferences of NEIL1 and NEIL2 raise the question about their affinity for various DNA structures. Our EMSA results with undamaged DNA showed that both NEIL1 and NEIL2 have higher affinity for bubble structures than for duplex DNA. Furthermore, the binding of NEILs to ssDNA was less specific than to the B11 bubble. These data strongly suggest that whereas NEIL1 and NEIL2 stably bind to single-stranded sequences even in the absence of substrate lesions, their initial interaction with DNA requires a duplex structure. The similar trend in binding of NEIL1 and NEIL2 with lesion-containing and nondamaged DNA in single-stranded, duplex, and bubble conformation supports our conclusion that these enzymes have intrinsic affinity for the bubble structure.

We then assessed the contribution of the base lesion to the binding by using inactive mutants of NEIL1 and NEIL2 with 5-OHU-containing oligos in various DNA structures. We observed a lower affinity of the mutants for the substrate oligo compared with that of the WT enzymes for the undamaged oligo (Table III). It thus appears likely that significant changes in the tertiary structures of these enzymes were induced by the mutations. Nevertheless, the affinity of both NEIL1 and NEIL2 for the bubble DNA was consistently higher than for the duplex DNA. This is in strong contrast to the situation with NTH1 and OGG1 which did not produce stable complexes with either ssDNA or B11 bubble.

Tainer and co-workers (33) have proposed a push-pinch-pull model for lesion detection by DNA glycosylases while scanning by NEILs along the DNA backbone. It is unclear how such a
model could apply to initial scanning when the duplex DNA changes abruptly to ssDNA before reaching the lesion. This issue may be resolved once the structures of DNA-bound NEILs are elucidated. Given the absence of requirement for ATR whether NEIL1 and NEIL2 move unidirectionally on the DNA strand is not known. Furthermore, it is highly likely that these enzymes, like other DNA glycosylases, flip out the damaged nucleotide before catalysis (34, 35). It should thus be easier for them to insert the base lesion into the catalytic pocket from ssDNA or a loop than from a duplex DNA. The inability of OGG1 and NTH1 to similarly utilize ssDNA or bubble DNA substrates may be due to distinct mechanisms involved in stabilizing the enzyme-substrate complex for the NEILs versus other DNA glycosylases.

Finally, the physiological implications of the unusual substrate structure preference of NEILs deserve to be discussed. The bubble DNA containing noncomplementary bases used in these studies was designed to represent unwound duplex regions in the genome. Similar bubbles are transiently formed in vivo during both transcription and DNA replication. Although preferential repair of DNA template strands during DNA replication has not been established, we had earlier hypothesized about replication-associated repair of mutagenic bases in mammalian cells (36). During DNA replication, several distinct DNA helicases function in unwinding the DNA duplex ahead of the replication fork to generate a bubble structure (37). S-phase-specific activation of NEIL1 raises the possibility that it is involved in repair of the damage present in the replication bubble.

In contrast, NEIL2, independent of cell cycle expression, could be involved in TCR. TCR of bulky base adducts (e.g. UV photoproducts), which prevent RNA chain elongation, was identified as a distinct subpathway of NER. The blockage of the transcription bubble. After base excision by NEIL2 in the transient bubble, generated ahead of the growing RNA chain, the DNA strand is cleaved due to the AP lyase activity of the enzyme. In contrast, OGG1 and NTH1 are unable to carry out base excision in the bubble. This could explain their dispensability in repairing damage from the transcribed sequences. The single-strand break generated by NEIL2 prevents forward movement of the transcription complex and induces either its retrograde movement or dissociation from the template (38). The bubble then collapses to reform the duplex structure, allowing completion of the strand break repair and resumption of transcription.

Whether NEILs interact with transcription and replication complexes in order to carry out targeted repair of DNA templates in a coordinated fashion remains to be investigated.

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