The Phosphodiester Bond 3′ to a Deoxyuridine Residue Is Crucial for Substrate Binding for Uracil DNA *N*-Glycosylase†

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ABSTRACT: Using the method of water-soluble carbodiimide-induced chemical ligation, four 27-member oligodeoxyribonucleotides containing a pyrophosphate internucleotide bond near or adjacent to a deoxyuridine residue were prepared. *Escherichia coli* uracil DNA *N*-glycosylase (UDG) activity was found to be sensitive to the presence of an internucleotide pyrophosphate bond in both single- and double-stranded DNA. The rate of uracil excision from single-stranded DNA containing a pyrophosphate bond adjacent to the uracil residue, either 3′ or 5′, was 0.01% and 0.1% of the rate of uracil removal from control DNA without a pyrophosphate bond, respectively. The rate of uracil excision from duplex DNA containing a pyrophosphate bond 3′ or 5′ to the uracil residue was also reduced, being 0.1% and 1% the rate of uracil removal from the corresponding duplex DNA control. Placing the pyrophosphate bond one nucleotide 5′ or 3′ away from the deoxyuridine in both single- and double-stranded oligodeoxyribonucleotides provided much better substrates for UDG. Kinetic measurements showed that the pyrophosphate bond placed adjacent to the deoxyuridine residue drastically reduced the affinity of UDG toward the modified DNA substrate, with the greatest effect occurring when the pyrophosphate bond was 3′ adjacent to the deoxyuridine. The enzyme was able to excise a 3′-terminal uracil at the nicked site of a nicked duplex DNA, provided that the terminal deoxyuridine was 3′-phosphorylated. The effect of the pyrophosphate bond on the substrate susceptibility of oligonucleotides containing deoxyuridine is discussed with respect to the mechanism of action of UDG.

In bacteria, simple base modifications to DNA have been shown to be repaired by the base excision repair pathway (Weiss & Grossman, 1987; Wallace, 1988; Lindahl, 1992). The initial step in the base excision repair pathway involves a class of enzymes called DNA *N*-glycosylases, which hydrolyze the glycosylic bond between the modified base and the sugar moiety. Two types of DNA *N*-glycosylases are found in *Escherichia coli*. Endonucleases III (Breimer & Lindahl, 1984; Kow & Wallace, 1987; Doetsch & Cunningham, 1990) and VIII (Melamede et al., 1994) and formamidopyrimidine DNA *N*-glycosylase (O'Connor & Laval, 1989; Doetsch & Cunningham, 1990) are *N*-glycosylases with an associated AP lyase activity. After removing the damaged base, this class of *N*-glycosylase will further catalyze the cleavage of the phosphodiester bond 3′ to the resulting abasic (AP) site by a β-elimination reaction (Bailly & Verly, 1987; Kow & Wallace, 1987; Kim & Linn, 1988; Doetsch & Cunningham, 1990; Mazumder et al., 1991). In contrast, uracil (Lindahl, 1977; Sakumi & Sekiguchi, 1990), Hypoxanthine (Karran & Lindahl, 1980; Harosh & Sperling, 1988), and 3-methyladenine (Bjelland & Seeberg, 1987) DNA *N*-glycosylases do not have an associated DNA cleavage activity and leave the DNA backbone intact but containing an AP site.

Uracil in DNA can arise from deamination of cytosine (Shapiro & Klein, 1966; Lindahl & Nyberg, 1974) or incorporation of dUTP by DNA polymerase (Bessman et al., 1958). Uracil derived from deamination of cytosine is highly mutagenic and results in C to T transitions (Duncan & Weiss, 1982). In fact, *E. coli* lacking uracil DNA *N*-glycosylase (UDG) has a high spontaneous mutation frequency (Duncan & Miller, 1980). Most repair enzymes from *E. coli* exhibit catalytic activities on double stranded DNA (Weiss & Grossman, 1987; Wallace, 1988; Doetsch & Cunningham, 1990; Lindahl, 1992); however, UDG is unique insofar as it removes uracil from both single- and double-stranded DNA (Lindahl et al., 1977; Sakumi & Sekiguchi, 1990; Varshney & Van de Sande, 1991). UDG has a molecular mass of 26 000 daltons, and requires no cofactor for its activity (Lindahl et al., 1977; Sakumi & Sekiguchi, 1990). The enzyme is inhibited noncompetitively by its product, uracil, as well as other uracil derivatives such as 6-aminouracil, 5-azauracil, and 5-fluorouracil (Caradonna & Cheng, 1980) and is able to remove 5-fluorouracil, but not 5-bromouracil or 5-methyluracil (thymine), from single- and double-stranded DNA (Warner & Rockstroh, 1980). Recently, UDG was shown to remove 5-hydroxydeoxyuridine (Hatahet et al., 1994) and 5,6-dihydroxydeoxyuridine from DNA (Zastawny et al., 1995). This observation thus raises question as to the specificity of the binding site of UDG toward uracil (Mol

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et al., 1995; Savva et al., 1995). Interestingly, the enzyme cannot remove a uracil residue from the 3′-terminus irrespective of the length of the DNA, and can only remove uracil from the 5′-terminus if the deoxyuridine is 5′-phosphorylated (Varshney & Sande, 1991).

The use of modified DNA is an established means for studying the substrate specificity as well as the mechanism of action of repair enzymes. Since many repair endo- and exonucleases require the cleavage of phosphodiester bonds, the introduction of a modified internucleotide linkage into the DNA backbone should provide uncleavable substrates for use in enzyme mechanism studies. For example, it has been shown previously that *Eco*RI and *Eco*RII restriction endonucleases are able to bind to restriction sites containing a pyrophosphate bond, but are unable to hydrolyze it (Purmal et al., 1984a,b, 1992). It has also been shown that the introduction of a pyrophosphate bond into DNA does not significantly affect the structure or thermal stability of double-stranded DNA (Dollinnaya et al., 1986). These data suggest that oligonucleotides containing such a bond could be useful as uncleavable substrates for the study of the binding properties of repair endonucleases to damaged DNA.

In the present work, we prepared a series of four 27-member oligonucleotides containing a unique pyrophosphate internucleotide bond instead of a natural phosphodiester bond either 5′ or 3′ to a unique deoxyuridine residue. We then examined the ability of *E. coli* UDG to remove uracil from these substrates in both single- and double-stranded forms and showed that DNA molecules containing a pyrophosphate bond either 3′ or 5′ to uracil are relatively poor substrates for UDG.

MATERIALS AND METHODS

Chemicals. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) and 2-(*N*-morpholino)ethanesulfonate (MES) were obtained from Aldrich; [γ-^{32}P]-ATP (>5000 Ci/mmol, 10 mCi/ml) was obtained from Amersham.

Enzymes. *E. coli* UDG was purified from *E. coli* strain BD 438 (Dr. B. K. Duncan, Duncan Laboratories, Philadelphia, PA) bearing overproducing plasmid pBD15 according to Lindahl et al. (1977) with modifications. The DNA−agarose step was omitted and replaced with FPLC chromatography on a Mono S column (HR10/10, Pharmacia) and further FPLC chromatography on a Mono Q column (HR5/5, Pharmacia) with a linear gradient of NaCl from 10 mM to 0.5 M. UDG was eluted from Mono S at 80−90 mM NaCl and from Mono Q at 0.15−0.18 M NaCl. The protein concentration was estimated to be 62.5 μg/mL (2.4 μM) using the Bio-Rad Protein Assay kit (Bio-Rad). T4 polynucleotide kinase was purchased from New England Biolabs; 3′-phosphatase-free T4 polynucleotide kinase was purchased from Boehringer Mannheim; *E. coli* alkaline phosphatase was obtained from Pharmacia LKB Biotechnology.

Oligonucleotides. The oligodeoxyribonucleotides [prefix "d" (deoxy) is omitted; lower case "p" denotes phosphoryl group] GGTCGACp, GGTCGACTp, GGTCGACTUp, GGTCGACTUAp, pTUAGGAGGATCCCCGGGTA, pUAGGAGGATCCCCGGGTA, pAGGAGGATCCCCGGGTA, pGGAGGATCCCCGGGTA, GGTCGACTUAGGAGGATCCCCGGGTA, and CCGGGGATCCTCCTAAGTCGACCTGCA were synthesized on an Applied Biosystems 380A DNA synthesizer (Department of Microbiology and Molecular Genetics, University of Vermont). The phosphorylating agent used for 5′- or 3′-phosphorylation of the oligonucleotides, 5′-*O*-DMT-deoxyuridine-3′-(2-cyanoethyl-*N*,*N*-diisopropyl)phosphoramidite, was purchased from Glen Research.

Oligonucleotides were purified by Mono Q (Pharmacia) ion-exchange chromatography on a Milton Roy HPLC equipped with a CM4000 Multiple Delivery system and a SM4000 Programmable UV Detector. The Mono Q column (Pharmacia, HR5/5) was equilibrated with buffer A (0.18 M NaCl, 0.01 M NaOH) at 1 mL/min. Crude oligonucleotides were bound to the column and then eluted with a linear gradient of 100% buffer A to 50% buffer A/50% buffer B (buffer B = 0.9 M NaCl, 0.01 M NaOH) over 50 min. After purification, oligonucleotides were desalted by gel filtration on NEP-5 or NEP-10 columns (Pharmacia) using water as an eluent.

Small amounts of 5′-phosphorylated oligonucleotides (2.5−5 nmol) were dephosphorylated with 0.01 unit of alkaline phosphatase in a buffer containing 50 mM Tris-HCl and 10 mM $MgCl_2$, pH 8.0. Dephosphorylated oligonucleotides were purified using the NENSORB 20 Nucleic Acids Purification Cartridge (Du Pont) following the manufacturer's instructions. Dephosphorylated oligonucleotides were then 5′-^{32}P-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase or 3′-phosphatase-free T4 polynucleotide kinase following standard procedures. Labeled oligonucleotides were further purified using a NENSORB 20 cartridge and combined with corresponding cold oligonucleotides to obtain the desired final specific radioactivity.

Synthesis of Oligonucleotides Containing a Pyrophosphate Internucleotide Bond. GGTCGACT**ppU**AGGAGGATCCCCGGGTA was prepared by carbodiimide-induced chemical ligation of GGTCGACTp and ^{32}pUAGGAGGATCCCCGGGTA as reported previously (Shabarova et al., 1981; Purmal et al., 1984a,b). Equimolar amounts (0.5 nmol each) of GGTCGACTp and ^{32}pUAGGAGGATCCCCGGGTA (10−50 nCi/0.5 nmol was used to detect the ligation product) were mixed with 1.5 times excess (0.75 nmol) of the template CCGGGGATCCTCCTAAGTCGACCTGCA in 5 μL of 50 mM MES-HCl, 20 mM $MgCl_2$, pH 6.0, buffer (MES-buffer). The mixture was then incubated at 80 °C for 2 min and cooled down to 4 °C over a period of 1 h. To the cooled mixture was added 5 μL of 0.4 M 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) in MES-buffer, and the template-mediated chemical ligation was allowed to proceed for 3 days at 4 °C in the dark (Shabarova et al., 1981; Purmal et al., 1984a,b). Periodically, 0.2 μL aliquots were removed and analyzed by electrophoresis in a 20% denaturing polyacrylamide gel containing 8 M urea followed by gel autoradiography. The yield following chemical ligation was 85−95% of the initial reactants. Similarly, oligonucleotide GGTCGACT**Upp**AGGAGGATCCCCGGGTA was obtained by template-mediated chemical ligation of GGTCGACT**Up** and ^{32}**p**AGGAGGATCCCCGGGTA; GGTCGAC**ppTU**AGGAGGATCCCCGGGTA was obtained from GGTCGAC**p** and ^{32}**pTU**AGGAGGATCCCCGGGTA; GGTCGACT**UApp**GGAGGATCCCCGGGTA was obtained from GGTCGACT**UAp** and ^{32}**p**GGAGGATCCCCGGGTA. In all cases, the same oligonucleotide-template, CCGGGGATCCTCCTAAGTCGACCTGCA, was used.

Single-stranded oligonucleotides with an internucleotide pyrophosphate bond were purified by electrophoresis in 1.5

mm thick 20% PAGE containing 8 M urea with subsequent electroelution from the gel. The purified oligonucleotides were then 5′-^{32}P-relabeled with [γ-^{32}P]ATP using T4 polynucleotide kinase following standard procedures (Chaconas & Van de Sande, 1980).

Double-stranded DNA containing a pyrophosphate bond was prepared by mixing together aqueous solutions of 5′-^{32}P-labeled modified oligonucleotides (2−3 pmol) with 1.5 times excess of template (CCGGGGATCCTCCTAAGTCGACCTGCA, 3−4.5 pmol). The mixture was evaporated to dryness in a Speed Vac Concentrator (Savant), redissolved in 9 μL of 10 mM Tris-HCl, 1 mM EDTA, and 10 mM NaCl, pH 7.5 (TEN), buffer, and incubated for 2 min at 65 °C. The solution was then allowed to cool down slowly to room temperature over a period of 1 h. The final concentration of double-stranded oligonucleotides was 0.2−0.3 μM.

Analysis of Products of the UDG Reaction. Appropriately diluted UDG (1 μL) was added to 4 μL of single- or double-stranded oligonucleotide (0.5 pmol) in TEN buffer. The reaction mixture was incubated for 15 min at 37 °C. The AP sites generated after the *N*-glycosylase reaction were cleaved by heating the reaction mixture at 90 °C for 30 min. The contents of the tube were mixed with an equal volume of loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol) and electrophoresed on a 20% polyacrylamide gel containing 8 M urea (0.4 mm thickness). The gel was dried and then autoradiographed for 8−10 h at −70 °C using intensifying screens and Fuji RX film. The radioactivity in the dry gel was then quantified with a BioRad PhosphoImager.

Steady-State Kinetic Analysis of the UDG Reaction. Kinetic constants for each of the single- and double-stranded DNA substrates were determined in 5 μL reactions containing various substrate concentrations (20 nM−1 μM) and appropriately diluted UDG in TEN buffer. Each of the reaction mixtures was incubated for 1, 2, and 5 min at 37 °C, so that an approximation of the initial velocity for UDG for each of the substrates could be obtained. The enzyme reaction was stopped by adding an equal volume of loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol). The AP sites generated after the *N*-glycosylase reaction were cleaved by heating the reaction mixtures at 90 °C for 30 min. Each of the reactions was then electrophoresed on a 20% polyacrylamide gel containing 8 M urea (0.4 mm thickness). The gel was dried, and the radioactivity in the gel was then quantified with a Bio-Rad PhosphoImager. Kinetic data were then analyzed using the Macintosh program k.cat (Biometallics, Inc.).

RESULTS

Properties of Oligonucleotides Containing an Internucleotide Pyrophosphate Bond. In order to study the substrate specificity of UDG, four oligonucleotides, each containing a unique 3′,5′-pyrophosphate bond placed at various positions with respect to the deoxyuridine residue, were prepared:

GGTCGACT**ppU**AGGAGGATCCCCGGGTAC oligo-ppU
GGTCGACT**Upp**AGGAGGATCCCCGGGTAC oligo-Upp
GGTCGAC**ppTU**AGGAGGATCCCCGGGTAC oligo-ppTU
GGTCGACT**UApp**GGAGGATCCCCGGGTAC oligo-UApp

All four pyrophosphate-modified oligonucleotides were synthesized using template-mediated chemical ligation, i.e., the covalent joining of oligonucleotides on a complementary template by the action of chemical agents (Shabarova, 1988). Oligo-ppU was obtained by the condensation of GGTCGACT**p** with **pU**AGGAGGATCCCCGGGTAC, oligo-Upp by the condensation of GGTCGACT**Up** with **p**AGGAGGATCCCCGGGTAC, oligo-ppTU by the condensation of GGTCGAC**p** with **p**T**U**AGGAGGATCCCCGGGTAC, and oligo-UApp by the condensation of GGTCGACT**U**Ap with pGGAGGATCCCCGGGTAC. In all cases, the same template, CCGGGGATCCTCCTAAGTCGACCTGCA, was used. The typical observed time course for template-mediated chemical ligation is shown in Figure 1. The rate of chemical ligation was about the same for all four oligonucleotides. After 3 days of incubation at 4 °C, the conversion of initial reactants to the product was approximately 90% (Figure 1, lane 6). It is important to note that in the absence of template, practically no ligation product was formed (data not shown).

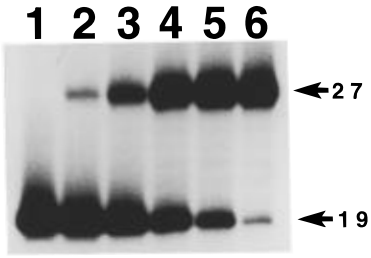

FIGURE 1: Time course of the carbodiimide-induced ligation of GGTCGACTp and ^{32}pUAGGAGGATCCCCGGGTAC. Electrophoretic analysis of the reaction mixture after the incubation of GGTCGACTp, ^{32}pUAGGAGGATCCCCGGGTAC, and a template strand (CCGGGGATCCTCCTAAGTCGACCTGCA) for 30 s (lane 1), 6 min (lane 2), 6 h (lane 3), 12 h (lane 4), 24 h (lane 5), and 48 h (lane 6) with EDC as described under Materials and Methods. The ligation product oligo-ppU (labeled as "27") and the precursor ^{32}pUAGGAGGATCCCCGGGTAC (labeled as "19") were detected by autoradiography.

The electrophoretic mobility of the 27-member oligonucleotides containing a pyrophosphate bond was slightly slower than for a 27-member oligonucleotide with a normal phosphodiester bond. This difference was noticeable only after 15−20 cm of migration into the gel. The pyrophosphate bond was stable to thermal breakage; heating oligonucleotides containing a pyrophosphate bond at 90 °C for 30 min in TE or TEN buffer did not yield any observable cleavage (data not shown).

Single-Stranded Oligonucleotides Containing a Pyrophosphate Bond Adjacent to or Near Deoxyuridine as Substrates for UDG. In order to study the effect of the pyrophosphate bond on the enzymatic activity of UDG, the pyrophosphate bond was placed in oligonucleotides at various positions relative to deoxyuridine. Oligo-ppTU, oligo-ppU, oligo-Upp, and oligo-UApp were incubated with different concentrations of UDG (50 pM−500 nM). Reactions were terminated after 15 min by adding the loading buffer followed by heating at 90 °C for 30 min. The heating not only inactivated the enzyme but also quantitatively cleaved the AP site (by the mechanism of β-elimination; Bailly & Verly, 1987; Kow & Wallace, 1987; Kim & Linn, 1988; Doetsch & Cunningham, 1990; Mazumder et al., 1991). The presence of a pyrophosphate bond in the vicinity of an AP site did not change the stability or the rate of heat-induced cleavage of the AP site as compared to a control AP site adjacent to a phosphodiester bond (data not shown). The reaction products were analyzed

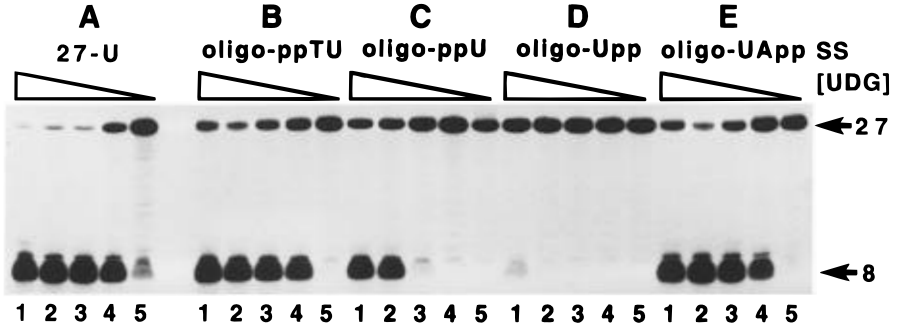

FIGURE 2: Comparison of uracil removal from single-stranded DNA substrates. 5′-^{32}P-Labeled GGTCGACTUAGGAGGATCCCCGGGTAC (27-U, panel A), oligo-ppTU (panel B), oligo-ppU (panel C), oligo-Upp (panel D), and oligo-UApp (panel E) were incubated with different amounts of uracil DNA glycosylase in TEN buffer for 15 min at 37 °C. The substrate concentration used for all reaction mixtures was 150 nM. The amounts of UDG used for each of the reactions were 480 nM (all panels, lane 1), 48 nM (all panels, lane 2), 4.8 nM (all panels, lane 3), 480 pM (all panels, lane 4), and 48 pM (all panels, lane 5). A right triangle in each of the panels indicates decreasing concentration of UDG. The oligonucleotide substrates are labeled as "27", and the reaction products are labeled as "8".

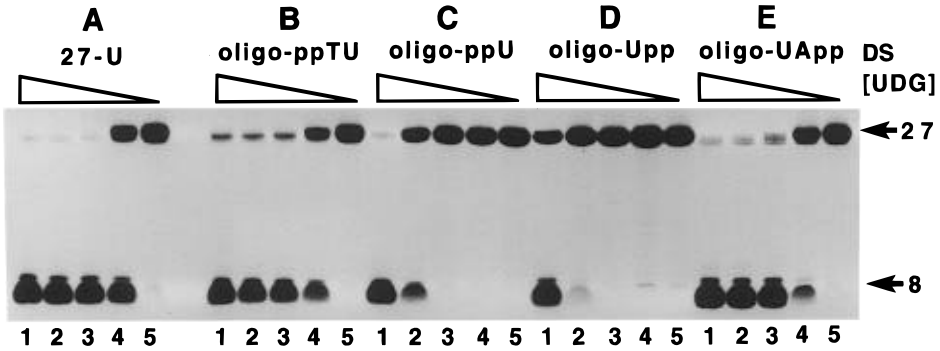

FIGURE 3: Comparison of uracil removal from double-stranded DNA substrates. 5′-^{32}P-Labeled GGTCGACTUAGGAGGATCCCCGGGTAC (27-U, panel A), oligo-ppTU (panel B), oligo-ppU (panel C), oligo-Upp (panel D), and oligo-UApp (panel E) were incubated with different amounts of UDG in TEN buffer for 5 min at 37 °C. The reaction conditions and amounts of UDG used were the same as in Figure 2). A right triangle in each of the panels indicates decreasing concentration of UDG. The oligonucleotide substrates are labeled as "27", and the reaction products are labeled as "8".

in a 20% denaturing polyacrylamide gel. The amount of 8-mer formed was then quantified with a Bio-Rad PhosphoImager, and this was then used to estimate the rate of uracil removal from each of the oligonucleotides.

Since UDG releases uracil from single-stranded DNA without hydrolyzing the phosphodiester bond (Lindahl et al., 1977; Sakumi & Sekiguchi, 1990), we expected that the presence of a pyrophosphate bond should not affect the rate of this removal. However, Figure 2 shows that the presence of a pyrophosphate bond adjacent to a deoxyuridine residue in a single-stranded DNA substantially reduced the rate of uracil removal. Using oligo-ppU (pyrophosphate bond 5′ to the deoxyuridine residue, Figure 2, panel C), approximately 0.1% of the rate of uracil excision was observed as compared to the normal substrate (Figure 2, compare panel A, lane 5, with panel C, lane 3). Placing the pyrophosphate bond 3′ to the deoxyuridine (oligo-Upp, Figure 2, panel D) further reduced the rate of uracil removal 10-fold, yielding a rate of about 0.01% (Figure 2, compare panel A, lane 5, with panel D, lane 1). However, when the pyrophosphate bond was placed one nucleotide either 5′ or 3′ away from the deoxyuridine (oligo-ppTU or oligo-UApp), the rate of removal of uracil was 80−90% (compare Figure 2, panel A and panel B) and 50−60% (compare Figure 2, panel A and panel E) that of the control substrate, respectively.

Duplex DNA Containing a Pyrophosphate Bond Adjacent to or Near Deoxyuridine as Substrates for UDG. In contrast to other repair enzymes, UDG can excise uracil residues from both single- and double-stranded DNA (Lindahl et al., 1977; Sakumi & Sekiguchi, 1990). For the *E. coli* enzyme, the rate of uracil excision is 2-fold higher for single- than double-stranded DNA (Lindahl et al., 1977; Sakumi & Sekiguchi, 1990). In agreement with this observation, we found that the rate of uracil removal from an unmodified single-stranded 27-member uracil-containing DNA was approximately twice that for double-stranded DNA (compare Figure 2, panel A, lane 4, with Figure 3, panel A, lane 4). However, the rate of uracil excision was slightly higher with double-stranded oligo-ppTU and oligo-UApp than with their single-stranded counterparts (compare Figure 2, panel B, and Figure 3, panel B, for oligo-ppTU; Figure 2, panel E, and Figure 3, panel E, for oligo-UApp), and approximately 5-fold higher in the cases of oligo-Upp and oligo-ppU (compare Figure 2, panel C, and Figure 3, panel C, for oligo-Upp; Figure 2, panel D, and Figure 3, panel D, for oligo-ppU).

When compared to the normal double-stranded substrate, the rate of uracil excision from double-stranded oligo-ppU and oligo-Upp was approximately 0.5 and 0.05% of the control, respectively (compare Figure 3, panel A, lane 4, with panel C, lane 2, and panel D, lane 1); however, for double-stranded oligo-ppTU and oligo-UApp, the rate of uracil excision approached about 40% that of the control (compare Figure 3, panel A, lane 4, with panel B, lane 4, and panel E, lane 4).

Kinetic Measurements of UDG Activity on DNA Substrates Containing a Pyrophosphate Bond. In order to further understand the interaction of UDG with these modified DNA substrates, the kinetic constants for UDG were measured for

Table 1: Kinetic Constants for UDG-Catalyzed Uracil Removal from Single-Stranded Oligonucleotides Containing a Deoxyuridine Residue and an Internucleotide Pyrophosphate Bond at Different Positions

substrates	K_m (nM)	V_{max} (nmol/min)	V_{max}/K_m	rel efficiency
27−5′-ppTU	69.7	38.4	0.55	0.58
27−5′-ppU	240.8	31.4	0.13	0.14
27-U	139.1	131.9	0.95	1
27-Upp-3′	591.9	24.5	0.04	0.04
27-UApp-3′	132.7	70.5	0.53	0.56

Table 2: Kinetic Constants for UDG-Catalyzed Uracil Removal from Double-Stranded Oligonucleotides Containing a Deoxyuridine Residue and an Internucleotide Pyrophosphate Bond at Different Positions

substrate	K_m (nM)	V_{max} (nmol/min)	K_m/V_{max}	rel efficiency
27−5′-ppTU	1.2	1.1	0.86	1.45
27−5′-ppU	19.5	3.8	0.19	0.32
27-U	4.5	2.7	0.59	1
27-Upp-3′	113.4	12.4	0.11	0.18
27-UApp-3′	3.7	2.5	0.68	1.10

each of the DNA substrates. The data are summarized in Tables 1 and 2. Table 1 shows that a pyrophosphate bond placed either 5′ or 3′ adjacent to the uracil residue decreases both the apparent binding of UDG (as indicated by an increase in the apparent K_m) to the DNA substrate as well as the apparent V_{max} of the enzyme toward the modified substrate. A large effect was observed when the pyrophosphate bond was placed 3′ adjacent to the deoxyuridine, causing a greater than 4-fold increase in the apparent K_m (from 139 nM to 591 nM) and a concomitant 5-fold decrease in the apparent V_{max}. A much lesser effect was observed when the pyrophosphate bond was 5′ immediate to the lesion; a 1.7-fold increase in the apparent K_m (from 139 nM to 241 nM) and a 4-fold decrease in the apparent V_{max} were observed. When the pyrophosphate bond was placed one nucleotide away from deoxyuridine, the relative efficiency of uracil removal approached 60% of that observed for the normal DNA substrate. Interestingly, when the pyrophosphate bond was one nucleotide 5′ away from the deoxyuridine, there was an apparent increase in the binding of UDG to the modified substrate, as indicated by a 2-fold reduction in the observed K_m (from 139 nM to 70 nM). A similar apparent increase in the binding affinity for the modified DNA substrate was observed for duplex substrates as shown in Table 2 (from 4.5 nM to 1.2 nM). The apparent substrate binding by UDG to the modified double-stranded substrates was affected to a much greater extent than the single-stranded substrate. When the pyrophosphate bond was 3′ adjacent to the lesion, a 25-fold increase in the apparent K_m (from 4.5 nM to 113 nM) was observed. In contrast, the enzyme exhibited a 5-fold increase in the apparent V_{max} for DNA substrates containing a 3′-pyrophosphate adjacent to the deoxyuridine residue. For double-stranded substrates, a pyrophosphate bond 5′ to the deoxyuridine only increased the apparent K_m 4-fold, and little change was observed in the apparent V_{max}. Similarly to the single-stranded DNA substrate, when the pyrophosphate bond was placed one nucleotide away from the deoxyuridine residue, the resulting DNA substrate was as good as a substrate as the control DNA substrate. These data suggest that the effect of pyrophosphate modification is relatively short-range, with its major effect being exerted when it is placed 3′ adjacent to the uracil residue.

Nicked DNA Duplexes Containing 3′-Deoxyuridine at the Nicked Site as Substrates for UDG. UDG was previously shown to remove uracil from the phosphorylated 5′-terminus of single-stranded DNA, but not from the 3′-terminus (Varshney & Van de Sande, 1991). In order for the 3′-terminal uracil residue to be excised by UDG, the deoxyuridine has to be adjacent 5′ to a 3′-phosphorylated nucleoside or at least a 3′-phosphorylated deoxyribose (Varshney & Van de Sande, 1991). Based on this observation, we reasoned that binding to the nucleotide or phosphodiester bond 3′ to the deoxyuridine residue would be crucial for UDG action. This was corroborated by the kinetic results obtained for UDG when DNA containing a pyrophosphate bond 3′ to the deoxyuridine was used as a substrate. Accordingly, we expected that a nicked duplex DNA substrate containing a uracil residue at the 3′-terminus of the nicked site would be a substrate for the enzyme

In order to test this hypothesis, the following duplexes were prepared by annealing appropriate oligonucleotides (the dashes within the DNA sequences represent the phosphodiester bond in the strand opposite the nick):

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  32pGGTCGACTUp                                  Duplex A
ACGTCCAGCTGAA--TCCTCCTAGGGGCC

  32pGGTCGACTUp AGGAGGATCCCCGGGTAC               Duplex B
ACGTCCAGCTGAA--TCCTCCTAGGGGC

  32pGGTCGACTUp pAGGAGGATCCCCGGGTAC              Duplex C
ACGTCCAGCTGAA-- TCCTCCTAGGGGCC

  32pGGTCGACTU pAGGAGGATCCCCGGGTAC               Duplex D
ACGTCCAGCTGAA- TCCTCCTAGGGGCC

  32pGGTCGACTU AGGAGGATCCCCGGGTAC                Duplex E
ACGTCCAGCTGAA-TCCTCCTAGGGGCC
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Nanonucleotide ^{32}**p**GGTCGACT**Up** was prepared by 5′-phosphorylation of GGTCGACT**Up** with [γ-^{32}P]ATP and 3′-phosphatase-free T4 polynucleotide kinase. The kinase used appeared to have residual 3′-phosphatase activity since we found two spots of labeled material corresponding to oligonucleotides with and without 3′-phosphate in the ratio of 8:2 (Figure 4).

Since the predicted T_m for the duplexes formed with the nanonucleotide GGTCGACTU is about 23 °C, in order to increase the amount of nicked and partial duplexes formed with the nanonucleotide GGTCGACTU, the oligonucleotide duplexes were formed with 200 nM each of the single-stranded oligonucleotides. The presence of both a 3′-phosphate and a 5′-phosphate groups in the nick of duplex C has little influence on the thermal stability of the duplex formed (Purmal et al., 1984a,b). The oligonucleotide duplexes A−E were then incubated with UDG at 20 °C (a lower reaction temperature was used in order to maintain the double-strandedness of duplexes A−E) for 30 min, heated at 90 °C for 30 min, and loaded onto a denaturing polyacrylamide gel (Figure 4). It can be seen (Figure 4, lane 2) that the enzyme did not remove uracil from single-stranded ^{32}pGGTCGACTUp alone, nor did it remove uracil from

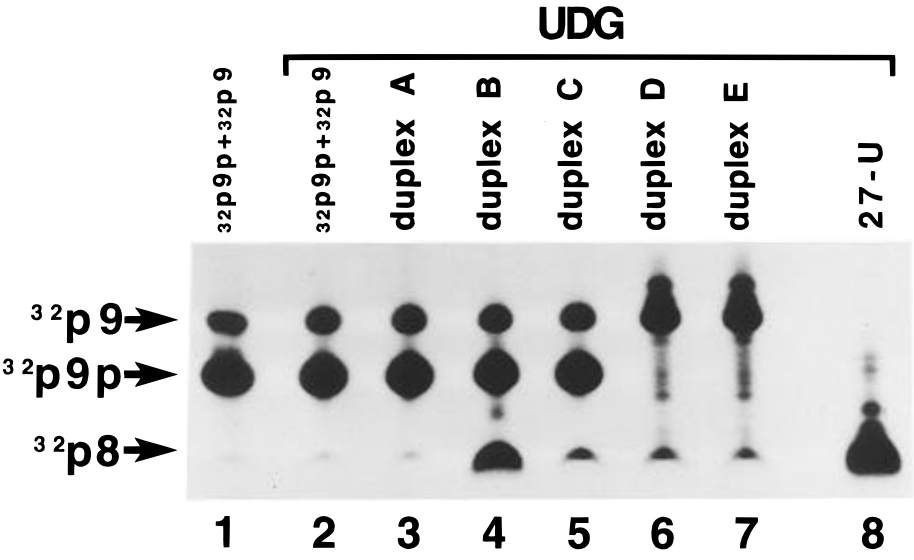

FIGURE 4: Comparison of uracil removal from nicked double-stranded DNA substrates. Electrophoretic analysis of reaction products obtained after treatment of duplexes A (lane 3), B (lane 4), C (lane 5), D (lane 6), and E (lane 7) with UDG (48 nM). The substrate concentration used in all reaction mixtures was 200 nM. Lane 1: ^{32}pGGTCGACT**U**p (labeled as "^{32}p9p") plus small amount of ^{32}pGGTCGACT**U** (labeled as "^{32}p9") with no enzyme. Lane 2: Single-stranded ^{32}pGGTCGACT**U**p and ^{32}pGGTCGACT**U** were treated with UDG. Lane 8: Control ^{32}pGGTCGACT**U**AGGAGGATCCCCGGGTAC (27-U) treated with UDG. All samples were heat inactivated by heating at 90 °C for 30 min before loading onto a 20% denaturing polyacrylamide gel for analysis.

^{32}pGGTCGACTUp hybridized with a complementary 27-member oligonucleotide (duplex A) (Figure 4, lane 3). However, when the putative "3′-binding site" (DNA chain 3′ to deoxyuridine residue) was provided in a double-stranded nicked complex by the oligonucleotide 3′ to the deoxyuridine residue as in duplexes B−E, different amounts of uracil were excised from the duplexes depending on the nature of the nick formed (Figure 4, lanes 4−7). It can be seen that a 3′-phosphoryl group immediate to the deoxyuridine is essential for substrate recognition. With excess amounts of UDG, 40% of the uracil can be removed from duplex B, which contained a 3′-phosphate at the nicked site (Figure 4, lane 4). However, nicked duplexes containing both 3′- and 5′-phosphates at the nicked site (duplex C, Figure 4, lane 5),containing only a 5′-phosphate (duplex D, Figure 4, lane 6), or lacking both 3′- and 5′-terminal phosphates (duplex E, Figure 4, lane 7) were extremely poor substrates for UDG (less than 0.5% of the substrates were converted).

DISCUSSION

Although all four oligonucleotides containing an internucleotide pyrophosphate bond were found to be substrates for *E. coli* UDG, the presence of a pyrophosphate 5′ or 3′ immediately adjacent to the deoxyuridine residue dramatically reduced the rate of enzymatic uracil removal. Less than 1% of the control rate of uracil excision was observed when the pyrophosphate bond was immediately adjacent to the deoxyuridine moiety (Figures 2 and 3, panels C and D). However, when the pyrophosphate bond was moved one nucleotide away from the deoxyuridine residue, almost all the reactivity was restored (50−80% of the control DNA). These data suggest that the binding of UDG to uracil-containing DNA predominantly involves only a trinucleotide, which is in good agreement with an earlier observation showing that the smallest DNA substrate for UDG is a 3′,5′-bisphosphate dinucleotide, pUpNp (Varshney & Van de Sande, 1991).

Steady-state kinetic measurements of UDG activity showed that the reduction in the rate of uracil removal from these modified DNA substrates is due to a large decrease in the binding affinity of UDG for the modified substrates, especially for DNA containing a pyrophosphate bond situated immediately 3′ to the deoxyuridine moiety (Table 2). For the double-stranded DNA substrate, the presence of a 3′-pyrophosphate bond reduced the apparent binding affinity of UDG by 25-fold as compared to only 5-fold reduction for a DNA substrate containing a 5′-pyrophosphate (Table 2). However, the decrease in the efficiency of uracil removal from these modified substrates by UDG was also partially due to an apparent decrease in the V_{max} values for the reaction, even though the latter effect is not as substantial as the changes in the apparent K_m values.

Based on the crystal structures solved for both human (Mol et al., 1995) and herpes simplex virus type 1 (HSV-1) UDG (Savva et al., 1995), two slightly different mechanisms of action for UDG were proposed. For the human enzyme, His-268 was suggested as the active site nucleophile for the cleavage of the N−C1′ glycosylic bond; in this case, a transient covalent enzyme−DNA intermediate would be formed between C-1′ of deoxyribose and the imidazole moiety of His-268. Once formed, uracil would be displaced, and the transient covalent intermediate then quickly hydrolyzed to yield the product, an AP site in DNA (Mol et al., 1995). Alternatively, attack on N−C1′ can be achieved by a water molecule activated by His-268, acting as a general base. In either case, Ser-270 was suggested to be important for interacting with the 3′ DNA sugar−phosphate backbone for stabilizing the His-268 imidazole position to effectively carry out the catalysis (Mol et al., 1995). The possible involvement of Ser-270 for binding was suggested by site-directed mutagenesis (Mol et al., 1995). An S270A mutant protein resulted in decreased DNA binding as well as a decrease in the rate of uracil hydrolysis, and is consistent with the idea that Ser-270 is important for the binding of the phosphodiester bond 3′ immediate to the deoxyuridine

moiety (Mol et al., 1995). A slightly different mechanism was proposed for the HSV-1 UDG (Savva et al., 1995). In this model, His-210 (which is equivalent to the His-268 for human UDG) would act as a general acid protonating the O-2 of the uracil base, thus weakening the N−C1′ glycosylic bond. Concomitant attack on C-1' by a water molecule, activated by Asp-88, would lead to the displacement of the uracil base. Based on the modeling for the binding of trinucleotide and uracil base by HSV-1 UDG, interaction of the 3′ end of the trinucleotide (or the 3′-phosphodiester bond) is essential for bringing the active site His-210 closed enough to protonate O-2 of deoxyuridine to achieve catalysis. Therefore, binding of the 3′-phosphoryl group by Ser-212 (Ser-270 in human) appears to be critical for catalysis. The mechanism of action described above for both the human and HSV-1 UDG suggested the importance of binding of UDG to the 3′-phosphodiester bond adjacent to the uracil moiety, and thus agrees with the steady-state kinetic results reported here. Kinetic measurements for UDG with the modified DNA substrates showed that changing the 3′-phosphodiester bond to a pyrophosphate bond predominantly reduces the apparent binding affinity of UDG for the modified DNA substrates. However, changing the 5′-phosphodiester bond to a pyrophosphate bond only modestly affected the apparent K_m for UDG (a 5′-pyrophosphate only caused a 5-fold decrease in K_m while a 3′-pyrophosphate reduced the K_m by 25-fold for double-stranded DNA substrates, Table 2).

The crucial role of the 3′-phosphodiester bond for UDG binding is further demonstrated by the observation that partial DNA duplexes containing a deoxyuridine at the 3′-terminus are not a substrate for UDG (Figure 4, lane 3). However, when the 3′ binding site was provided by hybridizing another DNA strand 3′ to the partial duplex, UDG reactivity was restored (Figure 4, lane 4). The rate of uracil removal from nicked duplex DNA containing uracil at the nicked site was 0.15% of the reaction rate with normal substrates (data not shown). It is important to note that in order for the nicked duplex to be a substrate for UDG, the 3′-deoxyuridine residue must be phosphorylated. The crystal structures of both the HSV-1 (Savva et al., 1995) and human (Mol et al., 1995) UDG revealed that UDG has a rigid structure, and that the active site does not undergo major conformational changes upon binding to DNA. The rigidity of the UDG structure may thus only allow binding of the 3′-phosphate, bringing the human His-268 (His-210 for HSV-1 UDG) residue into the correct orientation for catalysis. Removal of the 3′-phosphate would thus abolish binding of UDG to the DNA substrate, and eliminate catalysis (Figure 4, lane 7). Replacing the phosphate by putting it on the 5′-terminus of the nick may allow binding of UDG,but not the juxtaposition of the human His-210 (His-268 for HSV-1 UDG) residue for catalysis, again resulting in no catalysis (Figure 4, lane 6). These data suggest that UDG makes direct contact with the 3′-phosphate of the deoxyuridine residue. However, in addition to the 3′-phosphate, the presence of a 5′-phosphate at the nicked site might interfere with binding, thus reducing the UDG activity on the nicked substrate substantially (Figure 4, lane 5). This would be similar to the presence of a 3′-pyrophosphate bond in duplex DNA.

Changes in the observed V_{max} might be related to the recent common theme that is arising in the mechanism of action of several repair enzymes. Repair enzymes such as UDG (Savva et al., 1995; Mol et al., 1995), endonuclease III (Kuo et al., 1992), O^6-methylguanine−DNA methyltransferase (Moore et al., 1994), and T4 endonuclease V (Morikawa et al., 1995) were proposed to involve the flipping of the damaged base into an extrahelical position by the specific binding of the enzyme to DNA substrates. For UDG, it is possible that the presence of pyrophosphate bond in the vicinity of a deoxyuridine in a double-stranded DNA destabilizes the hydrogen bonding and stacking surrounding the uracil residue, thus decreasing the amount of activation energy required for flipping of the uracil base into an extrahelical position. The decrease in the activation energy is thus reflected as an apparent increase in the V_{max} for duplex DNA containing a pyrophosphate bond,either 3′ or 5′ immediate to the deoxyuridine residue (Table 2). However, for single-stranded DNA substrates, the flipping of the base might no longer be necessary; however, the presence of a pyrophosphate bond might interfere with the nucleophilic attack on the enzyme to the deoxyuridine residue, thus reducing the apparent V_{max} for the modified single-stranded DNA substrates (Table 1).

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